Formation of functional heterologous complexes using subunits from the picromycin, erythromycin and oleandomycin polyketide synthases

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Background: Recently developed tools for the genetic manipulation of modular polyketide synthases (PKSs) have advanced the development of combinatorial biosynthesis technologies for drug discovery. Although many of the current techniques involve engineering individual domains or modules of the PKS, few experiments have addressed the ability to combine entire protein subunits from different modular PKSs to create hybrid polyketide pathways. We investigated this possibility by *in vivo* assembly of heterologous PKS complexes using natural and altered subunits from related macrolide PKSs.

Results: The *pikAI* and *pikAII* genes encoding subunits 1 and 2 (modules 1–4) of the picromycin PKS (PikPKS) and the *eryAIII* gene encoding subunit 3 (modules 5–6) of the 6-deoxyerythronolide B synthase (DEBS) were cloned in two compatible *Streptomyces* expression vectors. A strain of *Streptomyces lividans* co-transformed with the two vectors produced the hybrid macrolactone 3-hydroxynarbonolide. Co-expression of the same *pik* genes with the gene for subunit 3 of the oleandomycin PKS (OlePKS) was also successful. A series of hybrid polyketide pathways was then constructed by combining PikPKS subunits 1 and 2 with modified DEBS3 subunits containing engineered domains in modules 5 or 6. We also report the effect of junction location in a set of DEBS–PikPKS fusions.

Conclusions: We show that natural as well as engineered protein subunits from heterologous modular PKSs can be functionally assembled to create hybrid polyketide pathways. This work represents a new strategy that complements earlier domain engineering approaches for combinatorial biosynthesis in which complete modules or PKS protein subunits, in addition to individual enzymatic domains, are used as building blocks for PKS engineering.

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Key words: combinatorial biosynthesis, macrolide, polyketide synthase

Received: **25 October 1999**
Accepted: **23 November 1999**

Published: **10 January 2000**

Chemistry & Biology 2000, **7**:77–84

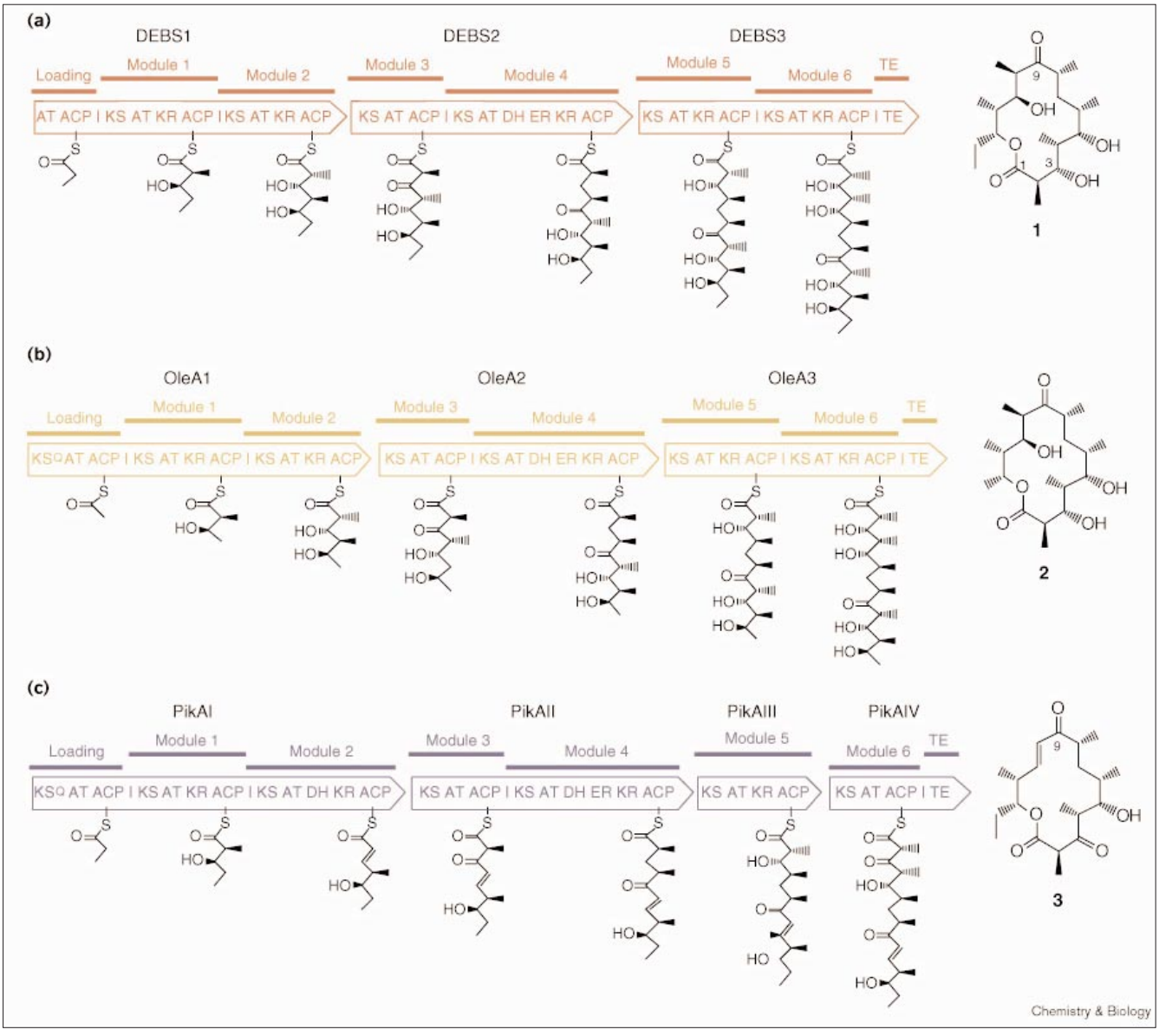
1074-5521/00/$ – see front matter

Introduction

Modular type I polyketide synthases (PKSs) catalyze the biosynthesis of macrolides and other macrocyclic polyketides, which have valuable medicinal properties. These multienzyme assemblies are formed by large multifunctional proteins that harbor a distinct active site for each step of catalysis in polyketide biosynthesis [1]. The active sites are arranged into repeated modules, with each subunit of the PKS complex containing from one to many (up to six have been reported) modules (Figure 1) [2]. The development of genetic engineering strategies that allow the structures of polyketides to be manipulated in ways beyond those accessible by conventional chemical approaches has utilized this convenient genetic and structural organization [3,4]. The direct correspondence between the catalytic domains present and the structure of the resulting biosynthetic product allows the polyketide structure to be altered by modifying the domains or modules of the PKS. This makes modular PKSs attractive targets for generating combinatorial polyketide libraries, as well as engineering analogs of compounds with demonstrated therapeutic activities [5,6].

The erythromycin PKS, 6-deoxyerythronolide B synthase (DEBS), from *Saccharopolyspora erythraea* has been the most widely used system for the development of such methods. Using DEBS, a number of experiments have shown that modular PKSs are amenable to a variety of targeted genetic modifications, such as inactivation, substitution or addition of domains or modules [2,7–20]. Recently, a combinatorial library of over 50 novel polyketides was prepared by direct domain engineering in DEBS [5]. Collectively, these experiments indicate that modular PKSs are fairly tolerant towards unnatural PKS substrates and that engineered modules maintain most of their structural integrity. But in a majority of the cases, protein engineering has resulted in reduced *in vivo* productivity. An alternative strategy for combinatorial biosynthesis is to assemble complete modules or protein subunits from heterologous sources. This approach would help alleviate

Figure 1



Modular arrangement of the related PKSs that produce **(a)** 6-deoxyerythronolide B (DEBS), **(b)** 8,8a-deoxyoleandolide (OlePKS), and **(c)** narbonolide (PikPKS). Each PKS consists of six modules, each of which catalyzes a round of condensation and subsequent β-keto modification on the growing polyketide chain. The colored modules highlight the differences in activities between each PKS system and the polyketide produced ([23,24]; R.M., unpublished observations). KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; TE, thioesterase; KS^Q, putative decarboxylation domain [32,33].

problems associated with structural imperfections that may be created by the engineered domains in mutant PKSs, including those reported in a previous study [5]. Favorable results obtained from recent functional module replacement experiments suggest that individual or groups of modules could also serve as functional units of manipulation for the construction of hybrid biosynthetic pathways [6,20]. Because many modules and subunits from different PKS systems are now available for genetic engineering, it is possible to examine their applicability towards combinatorial biosynthesis.

The oleandomycin PKS (OlePKS) from *Streptomyces antibioticus* and the picromycin PKS (PikPKS) from *Streptomyces venezuelae* are closely related to DEBS and have been completely sequenced ([21,22]; R.M., unpublished observations). The OlePKS produces a polyketide product, 8,8a-deoxyoleandolide (**2**), which differs from the product

of DEBS, 6-dEB (**1**), by only one carbon length in the priming unit. The product of the full-length PikPKS, narbonolide (**3**), differs from 6-dEB by a double bond at C-10,11, the lack of a methyl at C-10, and a ketone at C-3, all of which correspond to different activities or specificities of the active sites of modules 2 and 6, respectively. (The PikPKS also produces the 12-membered macrolactone, 10-deoxymethynolide, which results from premature cyclization of the module 5 intermediate [22,23].) We recently engineered a plasmid-based system for functional expression of PikPKS in the heterologous host *Streptomyces lividans*, which parallels the earlier described heterologous expression system used for DEBS [23,24]. Along with cloned subunits from OlePKS, these expression systems provide a convenient means for construction of heterologous PKS complexes *in vivo*, in which complete modules or larger protein units are manipulated. The compounds produced by all three PKSs are similar in size and functionalization and therefore comprise an ideal system to test whether polyketide intermediates can be passed between heterologous protein subunits while minimizing specificity barriers resulting from non-natural biosynthetic intermediates.

Here we report the successful construction of several heterologous PKS complexes *in vivo* generated by whole subunit complementation or intermodular fusions with these three modular PKS systems. The levels of polyketide production in bacteria containing these complexes were comparable to those obtained in the bacterial host containing only cognate subunits, and indicate productive association and transfer of biosynthetic intermediates between the heterologous PKS components. We further extend this application towards the development of combinatorial biosynthesis strategies by drawing from our existing repertoire of genetically modified DEBS subunits containing substituted domains from the rapamycin PKS (RAPS), which are capable of generating a diversity of biochemical features. The latter hybrid PKSs, containing heterologous PKS subunits as well as specific catalytic domain modifications, serve to illustrate the flexibility in engineering modular PKSs at many structural levels, including whole subunits, modules, catalytic domains, and various combinations.

Results and discussion

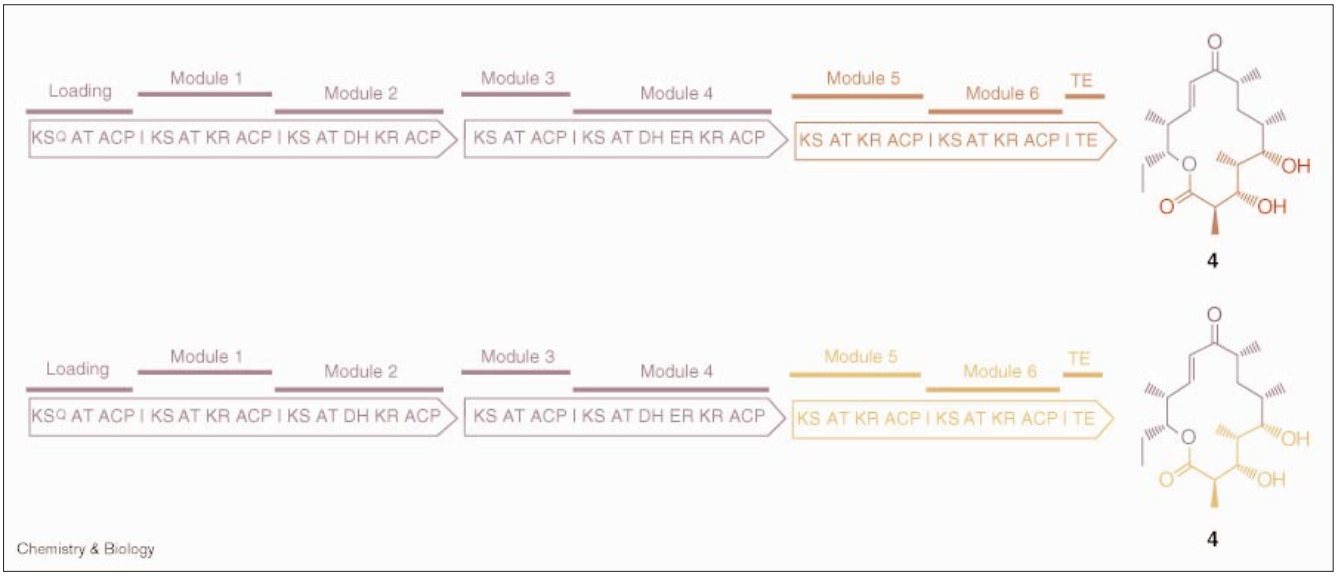
Hybrid PKS complexes with natural heterologous subunits

There are currently at least six modular PKSs belonging to the class of 12-, 14- and 16-membered macrolide antibiotics that have been sequenced at the DNA level. The compounds produced by these PKSs (erythromycin, picromycin/methymycin, oleandomycin, tylosin, niddamycin and mycinamicin) are structurally similar, but each contains one or more differences when compared to any other. One can therefore imagine designing 'hybrid' compounds incorporating the unique features from two or more pathways. Any structural permutation could, in theory, be produced by engineering the domains of each PKS scaffold as was done to construct macrolide analogs with DEBS. However, because the biosynthetic intermediates are similar between these PKSs, it is possible that modules and subunits from the different PKSs could functionally complement one another if they communicated productively.

We examined the feasibility of modular PKS subunit complementation by replacing PikAIII and PikAIV, the third and fourth subunits of the PikPKS, encoding modules 5 and 6, with DEBS3 and OleA3, each encoding modules 5 and 6 of their respective PKSs (Figure 2). The amino-acid identities between the pairs of subunits (considering PikAIII and PikAIV together as one subunit) are 42% for PikAIII/AIV:DEBS3 and also for PikAIII/AIV:OleA3. The DEBS3 and OleA3 subunits differ from PikAIII/AIV by the presence of a ketoreductase (KR) in module 6. Because there is only 46% identity between DEBS3 and OleA3, the subunits contain significant structural differences even though they are functionally equivalent. Based on the collective body of modular PKS engineering experiments, we expected that the DEBS3 and OleA3 subunits would process the unnatural intermediate generated by the PikPKS subunits. Whether the heterologous subunits would possess sufficient homology to associate, if required, or communicate properly had not been demonstrated, however.

We utilized the same bivector system developed for separate expression of DEBS subunits in *S. lividans* [25] to co-express PKS subunits from PikPKS with DEBS and OlePKS subunits. The *pikAI* and *pikAII* genes were placed on a single plasmid (pKOS039-83), whereas *er*y*AIII* (pKOS010-153) and *ole*AIII (labeled *ole*ORFB in [21]; pKOS039-133) were each placed on separate vectors compatible with pKOS039-83 (see Table 1). Expression of the genes is driven by the same promoter element, *act*Ip, and transcriptional activator, *act*II-ORF4, in all three plasmids. Plasmid pKOS039-83 contains the replicating *Streptomyces* origin from SCP2*, whereas plasmids pKOS010-153 and pKOS039-133 contain the ϕC31-*int-attP* loci for chromosomal integration [26]. Co-transformation of *S. lividans* K4-114 by pKOS039-83 and pKOS010-153 or pKOS039-133 using appropriate antibiotic selection resulted in strains capable of producing ~10 mg/l of the predicted hybrid macrolactone, 3-hydroxy-narbonolide (**4**) (referred to as 10-desmethyl-10,11-anhydro-6dEB in previous literature [5]; Figure 2) as determined using liquid chromatography/mass spectrometry (LC/MS) and nuclear magnetic resonance (NMR) spectroscopy. The amount of **4** produced was similar to levels of narbonolide (**3**) produced when the full set of PikPKS subunits was used [23]. It appears, therefore, that both the DEBS3 and OleA3 subunits fully complement the PikAIII and PikAIV subunits. For comparison,

Figure 2



Polyketides produced by complementation with heterologous PKS subunits from PikPKS (purple), DEBS (red), and OlePKS (orange).

the amount of **4** generated previously by replacement of the acetyltransferase (AT) and ketoreductase (KR) domains in module 2 of DEBS with RAPS cassettes was below 0.1 mg/l [5]. By employing naturally occurring subunits from different macrolide PKSs, we were able to effect a >100-fold improvement in yield of this compound.

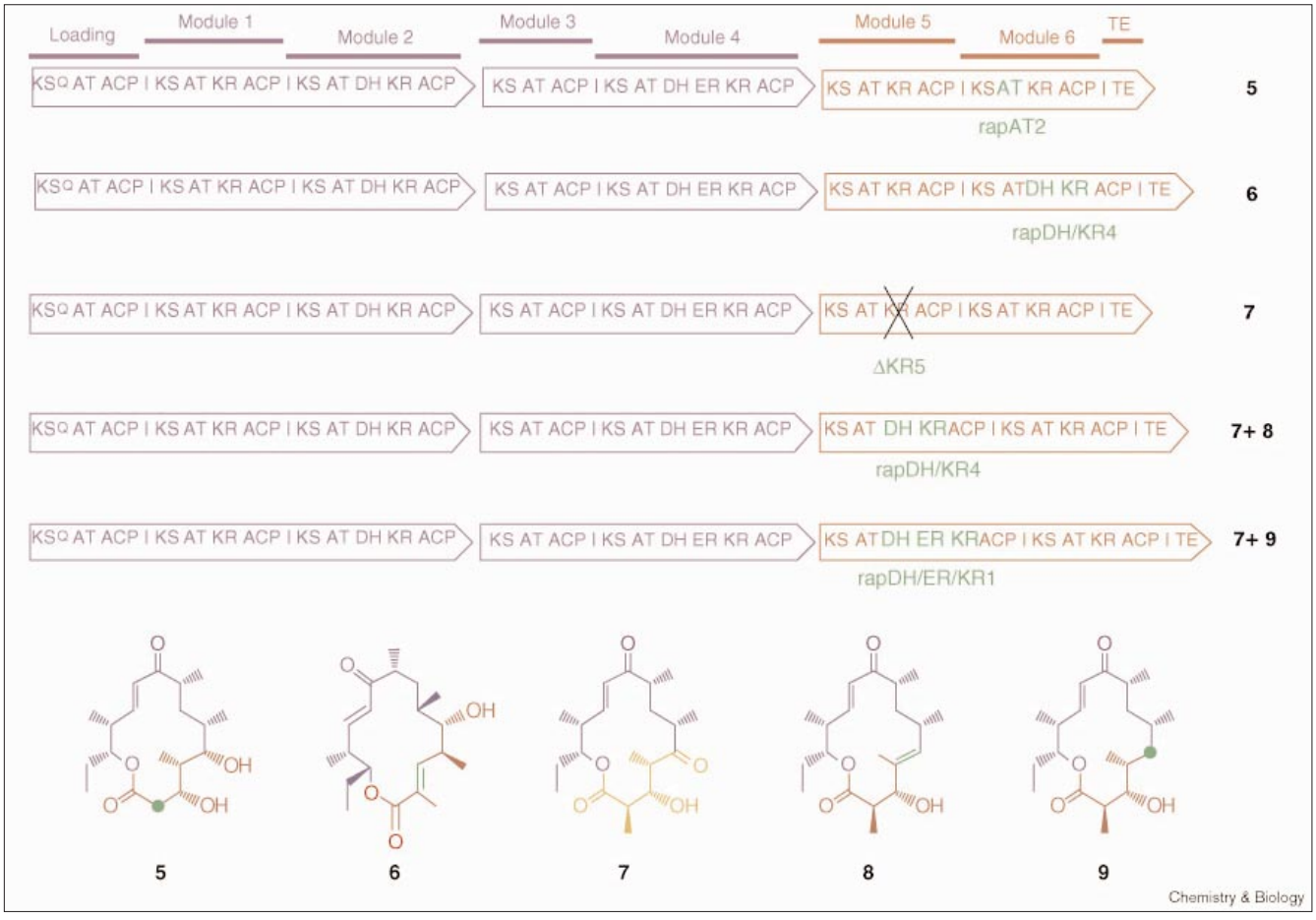
Hybrid PKS complexes with modified heterologous subunits

The construction of modular PKS subunits on separate expression vector systems holds certain advantages for generating combinatorial libraries. Once a set of subunits encoding different activities has been made, they may be rapidly combined via serial or co-transformation of a suitable host. This was recently accomplished with a set of modified DEBS subunits — containing domain deletions or substitutions with domains from RAPS — using three different expression vectors [27]. Because we had established that the DEBS3 subunit functions well with the PikAI and PikAII subunits, we decided to investigate whether the existing set of modified DEBS3 subunits could be used to propagate a series of narbonolide analogs. Because the expression plasmids encoding the modified DEBS3 were already available [27], it was convenient to now combine them with the above plasmid, pKOS039-83, in *S. lividans* using routine plasmid transformation.

Five DEBS3 subunits were selected, each containing a deleted or substituted domain in module 5 or 6 (Figure 3). These included a deletion of KR5 (pKOS025-1832), substitution of KR5 with rapDH/KR4 (pKOS025-1833), substitution of KR5 with rapDH/ER/KR1 (pKOS025-1834), substitution of KR6 with rapDH/KR4 (pKOS025-1841), and substitution of AT6 with rapAT2 (pKOS025-1842). Each of these is contained on the same integrating expression vector as the wild-type DEBS3 protein (pKOS010-153). All of the combinations produced at least one polyketide product as determined by LC/MS analysis (Figure 3). In the case of the two substitutions in module 6 and the KR5 deletion, the expected polyketides, 2-desmethyl-3-hydroxynarbonolide (**5**), 3-deoxy-2,3-anhydronarbonolide (**6**) and 5-deoxy-5-oxo-3-hydroxynarbonolide (**7**) were the major products. For the two domain substitutions in module 5, however, the expected compounds, 4,5-anhydronarbonolide (**8**) and 5-deoxynarbonolide (**9**), were detected in only trace amounts. Instead, the major compound in each case, 5-deoxy-5-oxo-3-hydroxynarbonolide (**7**), was formed presumably because the intermediate can efficiently bypass the incorporated RAPS domain. This was also observed when these mutations were introduced into module 5 of the complete DEBS system [5,27], suggesting that these RAPS domains do not operate optimally when inserted into module 5 of DEBS.

In the above experiments, a combination of subunit complementation and catalytic domain engineering was used to engineer the hybrid PKSs. Four of the five constructs were prepared using DNA derived from three different PKS gene clusters. This illustrates the remarkable range of flexibility that PKSs possess towards combinatorial engineering. Although many different permutations of subunits, modules and domains could be pieced together to generate a particular compound, clearly some combinations will result in more productive enzyme complexes. For example, in the previous DEBS library work, relatively few

Figure 3



Combinatorial library production by complementation with genetically engineered PKS subunits from PikPKS (purple), DEBS (red) and domains from RAPS (green).

macrolactones containing a 10-nor-10,11-anhydro moiety were generated because the domain modification in module 2 of DEBS (AT2→rapAT2, KR2→rapDH/KR4) produced only small amounts of 10-nor-10,11-anhydro-6-dEB [5]. We have extended the number of novel macrolactones with this functional arrangement by utilizing the naturally occurring set of activities found in the PikPKS modules.

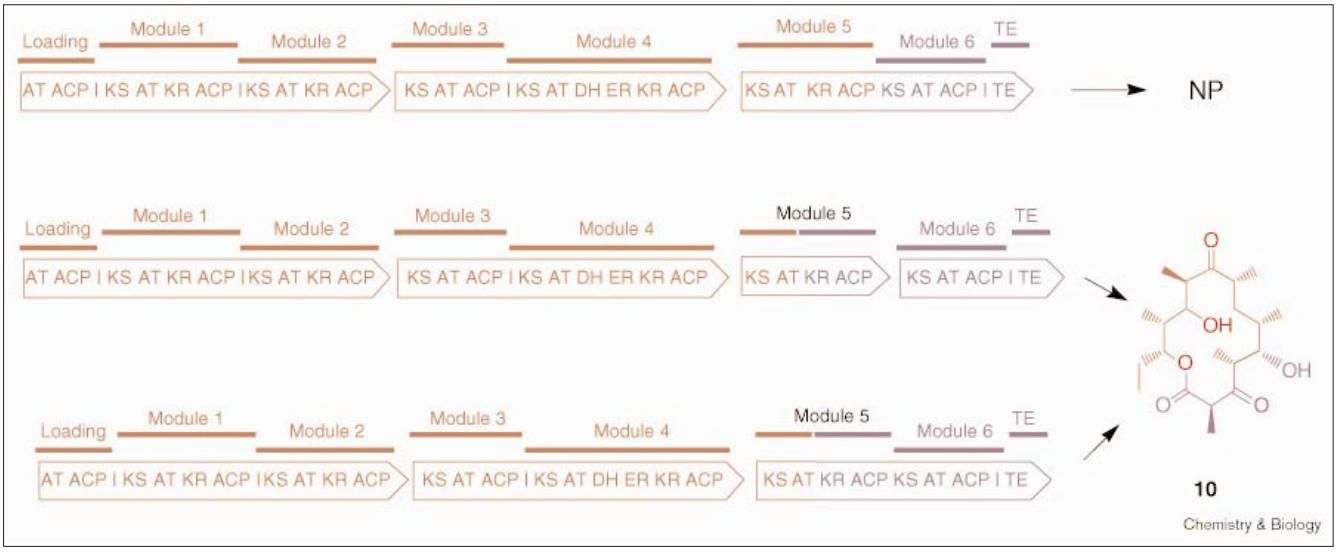
Hybrid pathways by intramodular fusions

The macrolides generated by the initial subunit complementation experiments above contain structural features unique to the early steps of narbonolide biosynthesis (no methyl at C-10, double bond at C-10,11) and the latter steps of 6-dEB biosynthesis (hydroxyl at C-3). Conversely, macrolactones with a methyl at C-10, hydroxyl at C-11 and a ketone at C-3 (i.e. 3-keto-6-dEB) would result from the early steps 6-dEB biosynthesis and latter steps of narbonolide biosynthesis. This compound has been produced previously by two different genetically engineered PKSs — a deletion of the KR domain in DEBS [5], and a fusion of the pikACP6 + TE to eryAT6 [23]. Recent success with functional module substitutions prompted us to test how PKSs engineered with complete module substitutions would compare with the other engineered mutant PKSs producing the same molecule.

The first attempt, in which module 6 of DEBS was replaced by module 6 of PikPKS (pKOS039-76, Figure 4), failed to produce any detectable polyketide. In this fusion, the conserved *Bsa*BI site at the start of DEBS KS6 was chosen for the junction site. The use of this conserved site led to highly productive intermodule fusions in a previous study by Gokhale *et al.* [20]. This hybrid PKS also maintained the DEBS M5-6 intermodular linker postulated to play a critical role in intermodular chain transfer and it is therefore unclear why no products were observed in this case.

Successful fusions between DEBS modules using junctions within modules (intramodular) rather than between

Figure 4



Engineered PKS fusions between DEBS (red) and PikPKS (purple). NP, no product.

modules (intermodular) have also been described previously [28]. It seemed reasonable that modules from heterologous PKSs could also be connected in a similar fashion. Two intramodular fusions were made between modules 5 of DEBS and PikPKS to create hybrid modules comprised of eryKS5 and AT5 domains, and pikKR5 and ACP5 domains (Figure 4). The PKS encoded by plasmid pKOS039-56 conserves the separate subunit architecture of the PikPKS, whereas plasmid pKOS039-89 encodes a PKS in which modules 5 and 6 are covalently linked.

S. lividans K4-114 transformed with plasmid pKOS039-56 produced ~3 mg/l of the expected macrolactone, 3,6-dideoxy-3-oxo-erythronolide B (**10**), verified by comparing chromatography profiles, mass fragmentation and ^{1}H NMR spectrum to an authentic sample [5]. The strain with plasmid pKOS039-89 also produced **10**, although yields were ~5–7-fold lower than those of the strain with plasmid pKOS039-56. It appears, therefore, that the engineered intermodular linker had an adverse affect on module assembly. The linker in this PKS was created by ligating two naturally occurring *Bsp*EI sites, resulting in the deletion of nine amino acids from the carboxyl terminus of module 5 and the first 22 amino acids of the amino terminus of module 6. This 'unnatural' linker might have disrupted proper folding or association between modules 5 and 6.

The difficulties encountered with the pKOS039-76 and pKOS039-89 constructs point towards the need to better understand the role of intermodular and interpolypeptide regions in the communication of PKS modules and subunits. However, the success of construct pKOS039-89 suggests that problems encountered with module fusions might be circumvented by constructing fusion junctions between the more highly conserved domains of modules, thereby preserving the natural intermodular or interpolypeptide linkers. A similar conclusion was reached in earlier work [28] and more recently by Ranganathan *et al.* [6], who produced a number of bimodular and trimodular hybrid PKSs with fusion junctions within modules. Together, the results reported here and elsewhere illustrate that a diversity of PKS engineering strategies from domain and module substitutions to subunit complementation and PKS fusions can be used for optimizing the production levels of unnatural polyketides.

Significance

Modular polyketide synthases (PKSs) that produce structurally similar compounds contain relatively minor differences in their genetic programming. Because the intermediates generated by the PKSs resemble each other, there is a high probability that the modules of one PKS will recognize and efficiently process the structurally related intermediates from another PKS. We demonstrate an approach to novel polyketide synthesis by complementation of subunits from related modular PKS systems. The advantage to this method is the structural conservation of naturally optimized protein subunits. We obtained high yields of 'unnatural' macrolide compounds when compared to previous genetic construction, which required engineering of individual domains within a module. This indicates that there can be good communication between noncovalent heterologous PKS modules. We also show that fusion junctions within modules can be useful for conserving potential

critical intermodular linker regions for constructing hybrid pathways.

These techniques serve two useful functions for generating potential therapeutic compounds. First, the ability to produce high yields of molecules that were produced at lower levels by other engineering methods increases the size of libraries made by combinatorial biosynthesis methods. This was shown by coupling the subunit complementation strategy with individual domain engineering to produce polyketide structures that were not accessible in previous library constructions. Second, it provides potential routes to high titers of hybrid compounds from structurally related families of polyketides, for example the 14- and 16-membered macrolide antibiotics (erythromycin, picromycin and tylosin), the polyketide immunosupressant family (FK506, FK520 and rapamycin), or the ansamycin-containing macrolides (rifamycin, geldanomycin and ansamitocin).

Materials and methods

Bacterial strains and media

S. lividans K4-114 [29] was the host used for expression of PKSs and production of polyketides. *S. lividans* transformants were selected on R2YE agar plates [30] using a 1 ml overlay of appropriate antibiotic, thiostrepton (1 mg/ml) or apramycin (2 mg/ml). *S. lividans* strains were grown in liquid R2YE (+50 mg/l thiostrepton) for production and analysis of polyketides. *Escherichia coli* XL1-Blue was used for DNA manipulation.

DNA manipulation

Transformation of *S. lividans* with plasmids was performed using the standard protoplast procedure [30]. For strains containing two expression plasmids, plasmids were introduced serially by transformation. Routine cloning was performed in Litmus28 (New England Biolabs) using standard procedures [31]. PCR was performed with *Pfu* polymerase (Stratagene) under conditions recommended by the manufacturer.

Construction of plasmids containing separate PKS subunits

Plasmid pKOS039-83, containing the *pikAI* and *pikAII* genes, is a derivative of plasmid pKOS039-86 [23] in which the *pikAIII* and *pikAIV* genes have been removed. It was constructed by first cloning a 15.68 kb *Bgl*II–*Pst*I fragment containing the *pikAII* gene and part of the *pikAI* gene into the corresponding sites of Litmus28. The fragment was then excised using *Bgl*II and *Xba*I and used to replace the 24.49 kb *Bgl*II–*Xba*I fragment in pKOS039-86 to generate pKOS039-83. Plasmid pKOS010-153 containing the *eryAIII* gene has been described previously [25]. Plasmid pKOS039-133 is a derivative of pKOS010-153 in which the *Nde*I–*Xba*I fragment containing the *eryAIII* gene has been replaced by the *oleAIII* gene (identified as ORFB in [21]) from plasmid pKOS039-132. Plasmid pKOS039-132 was constructed by excising the 10.8 kb *Asc*I–*Pst*I fragment of the *oleAIII* gene from cosmid pKOS055–1 (S. Shah, unpublished observations) and ligating with *Eco*RI–*Pst*I-digested Litmus28 and a DNA duplex linker created from the following two oligonucleotides: 5′-AATT*CATATG*GCTGAGGCGGAGAAGCTGCGCGAATACCTGT-GG-3′; 5′-CGCGCCACAGGTATTCGCGCAGCTTCTCCGCCTCA-GC*CATATG*-3′. The linker served to reconstruct the 5′ end of the *oleAIII* gene from the start codon to the *Asc*I site and introduce an *Nde*I site (in italics) at the start codon. Plasmids pKOS025-1832, pKOS025-1833, pKOS025-1834, pKOS025-1841 and pKOS025-1842, which contain the engineered *eryAIII* genes, have been described previously [27]. The plasmids used in this study are summarized in Table 1.

Table 1

Description of PKS expression plasmids used.

Plasmid	PKS gene encoded	Reference
pKOS039-83	*pikAI* and *pikAII*	This study
pKOS010-153	*eryAIII*	[25]
pKOS039-133	*oleAIII*	This study
pKOS025-1832	*eryAIII*, Δ*KR5*	[27]
pKOS025-1833	*eryAIII*, *ery*KR5→*rap*DH/KR4	[27]
pKOS025-1834	*eryAIII*, *ery*KR5→*rap*DH/ER/KR	[27]
pKOS025-1841	*eryAIII*, eryKR6→*rap*DH/KR4	[27]
pKOS025-1842	*eryAIII*, *ery*AT6→*rap*AT2	[27]
pKOS039-76	*eryAI*, *eryAII*, *eryAIII*/*pikAIV* fusion at the conserved *Bsa*BI site at *ery*KS6	This study
pKOS039-56	*eryAI*, *eryAII*, *eryAIII*/*pikAIII* fusion between *ery*AT5 and *pik*KR5, *pikAIV*	This study
pKOS010-89	Same as pKOS039-56 with fusion between end of *eryAIII*/*pikAIII* and start of *pikAIV*	This study

Construction of DEBS–PikPKS fusion plasmids

Plasmid pKOS039-56 contains a fusion between eryAT5 and pikKR5 at the previously engineered *Pst*I site [5] immediately downstream of eryAT5. The *pik* fragment containing the pikKR5-ACP and *pikAIV* was subcloned as a *Pst*I–*Eco*RI fragment and placed into pKAO127′kan′ [29] through a series of PCR and subcloning steps, resulting in the following DNA sequence at the fusion junction (*Pst*I site in italics): 5′-AGCG-GTACTGG*CTGCAG*AGCTCCGCGCC-3′. Plasmid pKOS039-89 is identical to pKOS039-56 except the 198 nt *Bsp*EI fragment between the end of pikACP5 and beginning of pikKS6 was deleted. Because the two sites are in-frame, this creates a single open reading frame in which modules 5 and 6 are translationally joined. Plasmid pKOS039-76 contains a fusion between the beginning of eryKS6 and pikKS6 located at the naturally occurring *Bsa*BI restriction site in eryKS6. The *pik* was subcloned as a *Bsa*BI/*Eco*RI fragment and placed into pKAO127′kan′ via a series of PCR and subcloning steps, resulting in the following DNA sequence at the fusion junction (*Bsa*BI site in italics) GGTGAGCC-*GATCGCGAT*CGTCGGCATGAG.

Analysis of polyketide products

S. lividans strains containing expression plasmids were grown in 2.5 ml liquid R2YE medium for 4 days at 30°C. Identification of polyketides in the supernatants was performed by LC/MS as described previously [27]. Quantity of polyketide production was determined by evaporative light scattering detection (ELSD). A previously characterized sample of 3,6-dideoxy-3-oxo-erythronolide B [5] was used to confirm the structure of **10**. Structural verification of 3-hydroxy-narbonolide (**4**) is described below.

*Purification and characterization of 3-hydroxy-narbonolide (**4**)*

Seed cultures (30 ml each of liquid R2YE medium) of *S. lividans* K4-114/pKOS039-83/pKOS039-133 were grown in eight flasks (250 ml) for 3 days at 30°C. Each seed culture was used to inoculate a 2 l flask containing 400 ml of liquid R2YE medium and grown for an additional 7 days. After centrifugation, the supernatants were combined and extracted with ethyl acetate (3 × 3 l). The solvent phase was dried over anhydrous $MgSO_4$ and the ethyl acetate evaporated. Partial purification was performed by silica gel chromatography (2 × 20 cm column, ethyl acetate:hexane = 1:2). Fractions containing 3-hydroxy narbonolide (**4**) were identified by LC/MS and pooled. Final purification was performed by HPLC using a C-18 reverse phase column (1 × 15 cm

column, 15–80% acetonitrile/water gradient over 45 min) to obtain 5 mg of pure **4**. The structure of 3-hydroxy narbonolide was confirmed by ^{1}H and ^{13}C NMR spectroscopy and HRMS. Assignment of chemical shifts was facilitated by HMQC–TOCSY experiments. ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm), 6.97 (dd, 1H, $J_1 = 4.68$ Hz, $J_2 = 16.24$ Hz, H-11), 6.11 (dd, 1H, $J_1 = 1.88$ Hz, $J_2 = 16.20$ Hz, H-10), 5.18 (m, 1H, H-13), 3.67 (dd, 1H, $J_1 = 1.60$ Hz, $J_2 = 8.92$ Hz), H-3), 3.52 (broad, 1H, H-5), 2.86(m, 1H, H-8), 2.64 (m, 1H, H-12), 2.57 (dq, 1H, H-2), 1.58-1.86 (m, 5H, H-6, H-7, H-12), 1.43 (m, 1H, H-4), 1.25 (d, 3H, $J = 6.8$ Hz, 2Me), 1.121 (d, 3H, $J = 8$ Hz, 12Me), 1.119 (d, $J = 6.8$ Hz 8Me), 1.01 (d, 3H, $J = 6.9$ Hz, 4Me), 0.95 (d, 3H, $J = 6.8$ Hz, 6Me), 0.94 (t, 3H, $J = 7.2$ Hz, 14Me). ^{13}C NMR (400 MHz, $CDCl_3$), δ (ppm); 204 (C-9), 176.1 (C-1), 150.1 (C-10), 126.7 (C-11), 77.2 (C-5), 76.3 (C-13), 75.2 (C-3), 44.1 (C-2), 42.0 (C-8), 50.6 (C-4), 39.0 (C-12), 35.7 (C-6), 35.4 (C-7), 19.3 (6Me), 17.9 (8Me), 14.9 (2Me), 10.4 (4Me), 10.3 (12 Me), 7.9 (15 Me). HRMS (FAB) calc'd for $C_{20}H_{34}O_5$ $[M + H]^+$ 355.248450; found 355.249010.

Acknowledgements

We thank Sejal Patel for assistance with compound purification, Mark Burlingame and John Carney for help with LC/MS and NMR spectroscopy, Sanjay Shah for providing cosmids containing the oleandomycin genes, Qun Xue for insightful discussions, Leonard Katz and C.R. Hutchinson for helpful comments on the manuscript.

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