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Genetic Approaches to Polyketide Antibiotics. 1

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1. Introduction

Microbial metabolites have for decades been a rich source of natural product drug leads and therapeutically important drugs.[1,2] The terms 'engineered biosynthesis' and 'combinatorial biosynthesis' encompass techniques aimed at increasing chemical diversity of natural products by altering the function of the genes and enzymes that govern the production of these metabolites. The classes of compounds most frequently associated with this are polyketides and nonribosomal peptides.[3] In particular, the predictable relationship between the structure and function of the modular type of microbial polyketide synthases (PKSs) has enabled genetic manipulation of the biosynthetic pathways for production of novel variants of classes of naturally occurring compounds, such as macrolide antibiotics[4,5] and antitumor compounds.[6] The goals of the approach resemble those of medicinal chemists who synthesize analogues and derivatives of lead compounds in an attempt to improve upon existing drugs or find new ones. Expression of native or engineered PKS genes, as well as those that govern precursor supply and post-PKS modification of the metabolite, in heterologous hosts is also an important aspect of developing commercial systems for drug production. The following review presents an overview of the initial technology enabling studies pertaining to polyketide manipulation (which have been covered in several previous reviews[4−11]) and a comprehensive review of the more recent advances that have set the stage for broader use of the technology. Although this review focuses on antibacterial polyketides, engineered or combinatorial biosynthesis extends to other classes of polyketides of therapeutic interest including antitumor, immunosuppressive, antifungal, and other compounds.

Polyketides are a notable class of natural products with a number of well-established successes in clinical and agricultural applications. Examples include the antibiotics erythromycin, tylosin, rifamycin, and the tetracyclines, immunosuppressants FK506 and rapamycin, and antitumor agents doxorubicin and mithramycin. Two early seminal works led to the promise that the genes for secondary metabolites could be used to create novel structures. The first was by Hopwood and co-workers[12,13] in which genes from two different antibiotic gene clusters, medermycin or granaticin with actinorhodin, were used in combination for the first time to produce a hybrid aromatic polyketide. The second was work performed by Leadlay and co-workers at Cambridge[14] and by Katz and co-workers at Abbot Laboratories,[15] who uncovered the first genes for a modular (type I) PKS in the erythromycin gene cluster. These megasynthases have since been the primary focus of polyketide engineering, and many of the proof of concept experiments have been performed using the erythromycin PKS.

Modular PKSs make many large and complex natural products that are difficult to synthesize or modify by chemistry. Since the discovery of the erythromycin PKS, on the order of a few hundred new compounds have been created through modification or heterologous expression of polyketide gene clusters. These numbers suggest that, in its current state, polyketide engineering is best suited for optimization of existing lead compounds by specifically chosen structural modifications rather than random

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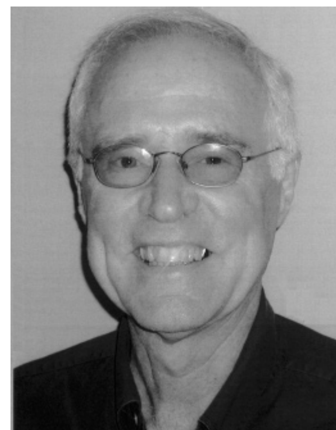
Robert McDaniel is a Senior Scientist at Kosan Biosciences, Inc. and has worked extensively in the field of polyketide biosynthesis and engineering for more than 12 years. He received his B.S. degree in Chemical Engineering from the University of Colorado at Boulder and M.S. and Ph.D. degrees in Chemical Engineering at Stanford University. At Stanford he began his career in polyketide research in the laboratory of Professor Chaitan Khosla, collaborating with Professor Sir David Hopwood at the John Innes Institute. He then joined Kosan during its genesis in order to transfer and further develop polyketide engineering technology that had been developed at Stanford. He has worked at Kosan for the past 8 years on several projects, including anti-infectives, and has published several research articles on the subject. His current interests are focused on ways to improve polyketide production from natural and engineered polyketide producing microorganisms.

Mark Welch has over 10 years experience in RNA and protein engineering with particular emphasis on directed evolution methods to create novel biocatalysts. Mark received his B.A. degree in Biology from the University of California at Santa Cruz with College Honors and Highest Honors in the Major. He received his doctoral degree in Molecular, Cellular, and Developmental Biology from the University of Colorado at Boulder in 1996 for studies of the role of 23SrRNA in the peptidyl transferase activity of the ribosome in the laboratory of Dr. Michael Yarus. In 1998 Mark joined Maxygen, Inc., a biotechnology company specializing in the application of DNA shuffling methods for biomolecule-directed evolution. As a scientist in the New Technology group at Maxygen, Mark managed an interdisciplinary team responsible for development and application of novel high-throughput assays to screen for a variety of target enzyme activities. He joined the New Technology group at Kosan in April 2003 and is currently leading a group focused of novel PKS engineering.



C. Richard (Dick) Hutchinson is a Research Fellow at Kosan Biosciences, Hayward, CA, following 3 years as the Vice President of New Technologies after joining the company in March 2000. He spent 26 years at the University of Wisconsin, Madison, as a Professor of Medicinal Chemistry and Bacteriology and is now a Professor Emeritus. Prior to this he was on the faculty of the School of Pharmacy, University of Connecticut (1970–74), conducted postdoctoral research with Sir Alan Battersby (1971), and received his Ph.D. degree in Organic Chemistry from the University of Minnesota (1970), working with Edward Leete, and B.S. degree in Pharmacy from Ohio State University (1966), where he carried out undergraduate research with Raymond Doskotch and Jack Beal. His research has involved studies of the biosynthesis of natural products with special emphasis on the molecular genetics and biochemistry of antibiotic production in microorganisms. He has published over 235 scientific papers and patents and received several prestigious awards, including Guggenheim and Fullbright fellowships, the Charles Thom Research Achievement Award of the Society for Industrial Microbiology, the AACP Paul Dawson Biotechnology Award, the Research Achievement Award of the American Society for Pharmacognosy, and a Distinguished Alumni Award from Ohio State University.

generation of large 'libraries' for screening against targets. Some of the applications for which this technology is used at Kosan include the following: (i) production of analogues of natural products to improve activity or pharmacodynamics; (ii) introducing reactive groups into compounds for further chemical modification; (iii) generating intermediates for chemical synthesis of natural products; and (iv) increasing the availability or reducing the cost of natural products (overproduction). Although the architecture of PKSs suggests that very large libraries could be theoretically generated, the challenges to doing this in the laboratory require a further understanding of PKS structure, specificity, and protein interactions as well as technologies to perform genetic manipulation more efficiently.

Other approaches to discovering new drugs from natural products are not discussed here but typically rely on high-throughput DNA sequencing. For example, novel sets of secondary metabolism genes are found by either whole genome sequencing or DNA libraries enriched for secondary metabolite genes and subsequently expressed through manipulation of growth conditions or in heterologous hosts. DNA libraries generated from unculturable organisms or environmental samples can also be screened in this manner or cloned directly into heterologous hosts and screened for biological activity. Finally, the structural modification of aromatic polyketides, made by so-called type II PKSs, is less amenable to gene manipulation and therefore is not reviewed here, except to note recent advances in understanding this area.

2. Biochemistry and Genetics of Polyketide Biosynthesis

Polyketide synthases evolved the capability of making a vast number of compounds from the same classes of substrates used by fatty acid synthases and

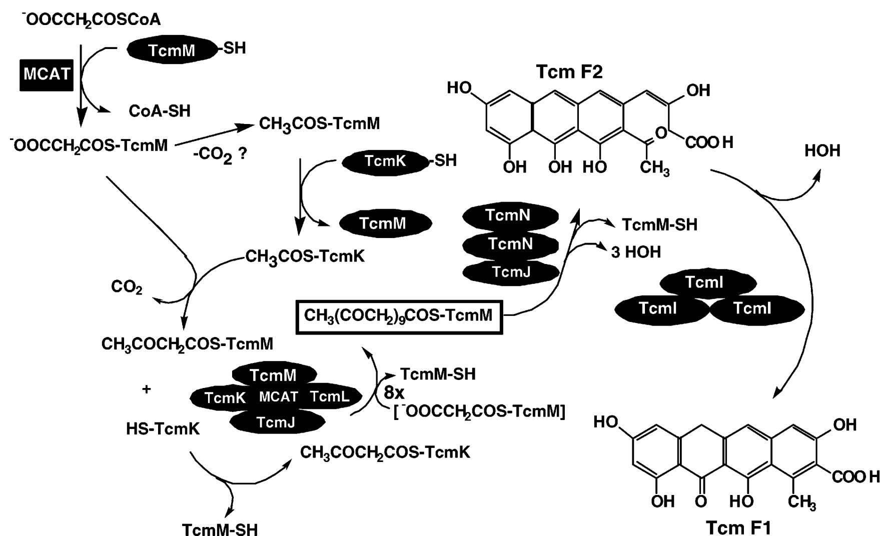


Figure 1. Illustration of the mechanism of the type II PKS involved in the biosynthesis of tetracenomycin F1 and C. The PKS consists of individual protein subunits that act in concert to assemble the acetate starter unit (produced by decarboxylation of enzyme-bound malonate) and nine chain extender units into a TcmM-bound decaketide by an iterative process involving a malonyl-CoA:ACP acyltransferase (MCAT, which is shared with fatty acid biosynthesis) and the proteins TcmJ, TcmK, TcmL, and TcmM. The decaketide is cyclized to Tcm F2 by the TcmN enzyme, with assistance by TcmJ; then Tcm F2 is cyclized once more by TcmI to form Tcm F1. The latter intermediate is converted to tetracenomycin C by tailoring enzymes.

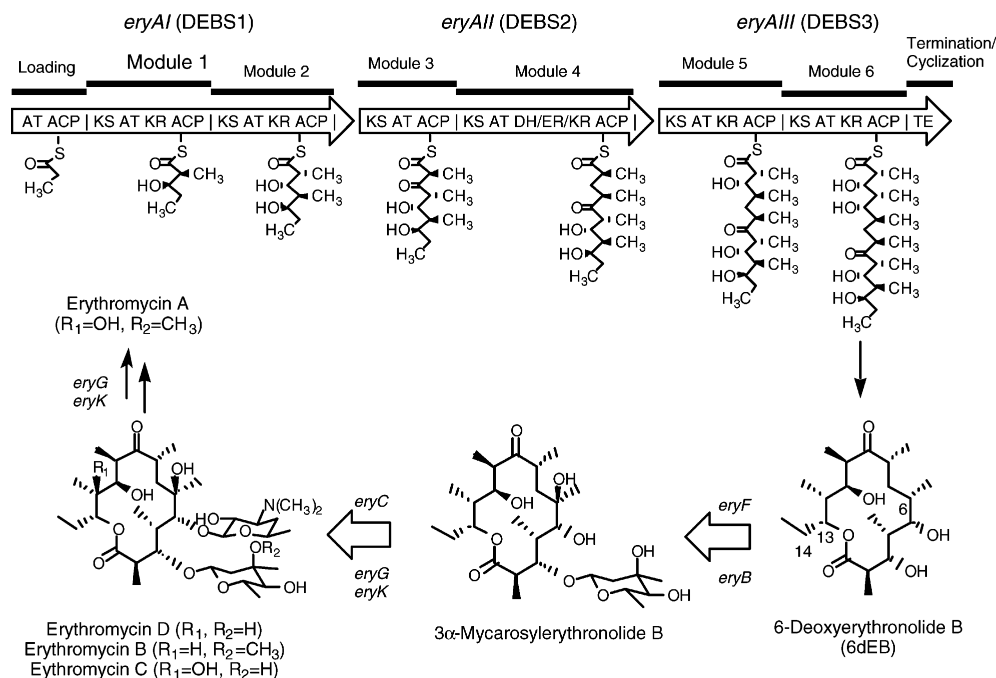


Figure 2. Illustration of the mechanism of the type I modular PKS involved in the biosynthesis of 6dEB. Each of the DEBS subunits is represented by a broad, open arrow containing the relevant domains in each module. Key enzyme-bound intermediates of carbon-chain assembly are shown bound to the ACP domains. Assembly begins at the loading didomain of the first DEBS subunit upon attachment of propionate which then reacts with ACP-bound 2-methylmalonate, obtained from its CoA ester. Further equivalents of 2-methylmalonyl-CoA are used by DEBS to produce 6dEB, as explained in the text. 6dEB is then converted to the erythromycin A–D glycosides by tailoring enzymes.

largely by the same type of biochemistry. Examples of two types of PKSs and their associated polyketides are illustrated in Figures 1 and 2. Type II, or aromatic, PKSs (Figure 1) consist of a collection of largely monofunctional proteins that catalyze the formation of typically polycyclic aromatic compounds, usually from acetate and malonate only. Type I, or modular, PKSs (Figure 2), in contrast, use large multifunctional proteins to make polyoxygenated, aliphatic compounds from several different kinds of acyl-Coenzyme A substrates.

The so-called modular PKSs[15–17] have led to the most fruitful genetic engineering route to structural variants[18,19] of polyketides that are important therapeutic drugs, like the antibacterial erythromycin A (Figure 2) or experimental agents such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) that currently is undergoing clinical trials as an antitumor

drug. [Two other types of PKSs, the fungal nonmodular type I and plant chalcone type III, are not discussed here; for reviews, cf. refs 20 and 21.] A modular PKS is a massive complex of large, multifunction proteins. Within each protein are one or more "modules", each with different combinations of domains that function like the constituent biochemical activities of fatty acid synthases to catalyze a single cycle of polyketide chain elongation and modification. 6-Deoxyerythronolide B synthase (DEBS) is the PKS that forms the backbone of the erythromycins and is encoded by the three genes, *eryAI–III*[14,15,22] (Figure 2). DEBS catalyzes formation of 6-deoxyerythronolide B (6dEB) by the successive condensation of one propionyl and six 2-methylmalonyl molecules in their activated Coenzyme A (CoA) thioester form. Each of the three subunits of DEBS have two extender modules, containing the activities needed for one cycle of polyketide chain elongation, as illustrated by the structures of the six enzyme-bound intermediates in Figure 2. In addition, the first module is preceded by a loading didomain for the starter unit, and the last is followed by a thioesterase domain for product release and cyclization. Every extender module contains a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain that together catalyze a two-carbon extension of the chain. In DEBS, the AT domains of extender modules are specific for 2-methylmalonyl-CoA, while the AT in the loading module uses propionyl-CoA. After each two-carbon unit condensation, the oxidation state of the β-carbon is either retained as a ketone (module 3) or modified to a hydroxyl, methenyl, or methylene group by the presence of a ketoreductase (KR) (module 2), a KR + a dehydratase (DH), or a KR + DH + an enoyl reductase (ER) (module 4), respectively.

In effect, the AT specificity and the types of catalytic domains within a module serve as codes for the structure of each two-carbon unit; the order of the modules in a PKS specifies the sequence of the distinct two-carbon units, and the number of modules determines the length of the polyketide chain. Variations in the acyl-CoA substrates used by a modular PKS, the number of domains within a module, and the number of modules in the PKS are responsible for establishing the first set of structural characteristics of the polyketide, including the chirality of hydroxyl- and alkyl-bearing carbon centers. After this, the kinds of biochemical transformations the compound produced by the PKS undergoes, such as glycosylation or oxidation, are dictated by the "tailoring enzymes" that establish the final structure. Consequently, engineering a microorganism to produce novel polyketides can involve altering only the PKS genes or the tailoring genes as well.

3. Polyketide Antibiotic Gene Clusters

3.1. Fourteen-Membered Macrolides

The 14-membered macrolides are exemplified by erythromycin, for which the complete gene cluster has been sequenced.[14,15,22–28] The complete or partial gene clusters for oleandomycin,[29–34] megalomicin,[35] and picromycin[36] are also known (Figure 3). The overall DEBS-like architecture of the PKS genes is conserved with the exception of the picromycin PKS (PicPKS) (Figure 4), in which the last two modules are contained on two separate proteins. Both DEBS and the megalomicin PKS (MegPKS) produce 6dEB and have high sequence similarity.[30] The oleandomycin PKS (OlePKS) produces 8,8a-deoxyoleandolide, which is equivalent to 6dEB derived from a two-carbon starter unit rather than a three-carbon starter unit. An important distinction between DEBS (and MegPKS) and OlePKS is the composition and mechanism of their loading domains. In DEBS, an AT loads propionyl-CoA (as well as other acyl CoAs with lower efficiency) whereas the OlePKS loading domain contains an AT specific for malonyl-CoA and a KS^q domain, an inactive condensation domain that serves to decarboxylate the ACP-bound substrate to acetyl-ACP for use as the starter.[30,37]

The PicPKS differs from the other three PKSs in several ways. The loading domain of the PicPKS also contains a KS^q domain but an AT that is specific for methylmalonyl-CoA, which is then decarboxylated to propionyl-CoA for use as the starter. Module 2 of the PicPKS extends using a malonyl-CoA rather than a methylmalonyl-CoA unit and also contains a DH domain in addition to the KR β-keto modifying activity leading to the C-10/C-11 alkene. Finally, module 6 lacks a KR, resulting in the 3-ketone of narbonolide. The PicPKS also produces approximately stoichiometric amounts of the 12-membered lactone precursor of methymycin, methynolide, via a mechanism referred to as domain skipping.[10,36,38,39]

All four PKSs have been expressed heterologously in *Streptomyces coelicolor* and/or *Streptomyces lividans* and their corresponding aglycones (**1–3**) produced in good yield.[30,35,38,40] The MegPKS contains two regions longer than 0.5 kb with 100% sequence identity in modules 2 and 6, leading to plasmid instability caused by homologous recombination during plasmid-borne heterologous expression.[41] Similarly, OlePKS contains over 1.2 kb of identical sequence in modules 2 and 5. Neither DEBS nor PicPKS possess such repeats.

The polyketide macrolactones are further modified by a series of glycosylation(s) and oxidation(s). Synthesis of 6dEB is followed by C6-hydroxylation by the product of *eryF* to yield erythronolide B. Addition of the sugar L-mycarose (via thymidine diphospho-(TDP)-mycarose) yields 3-*O*-α-mycarosylerythronolide B, and the addition of D-desosamine (via TDP-desosamine) yields erythromycin D. The two sugars are produced by independent sets of genes designated *eryB* (mycarose) and *eryC* (desosamine), which flank the PKS genes in the cluster. The final steps of erythromycin biosynthesis are hydroxylation of erythromycin D to yield erythromycin C by a second P450 enzyme encoded by *eryK* and *O*-methylation of the mycarosyl residue by the *eryG* product to yield the cladinosyl moiety of erythromycin A and B. Megalomicin tailoring resembles that of erythromycin except that a third deoxysugar, L-megosamine, is added to the C-6 hydroxyl and mycarose is acylated at C-3‴ and C-4‴. The set of genes for both TDP-L-mego-

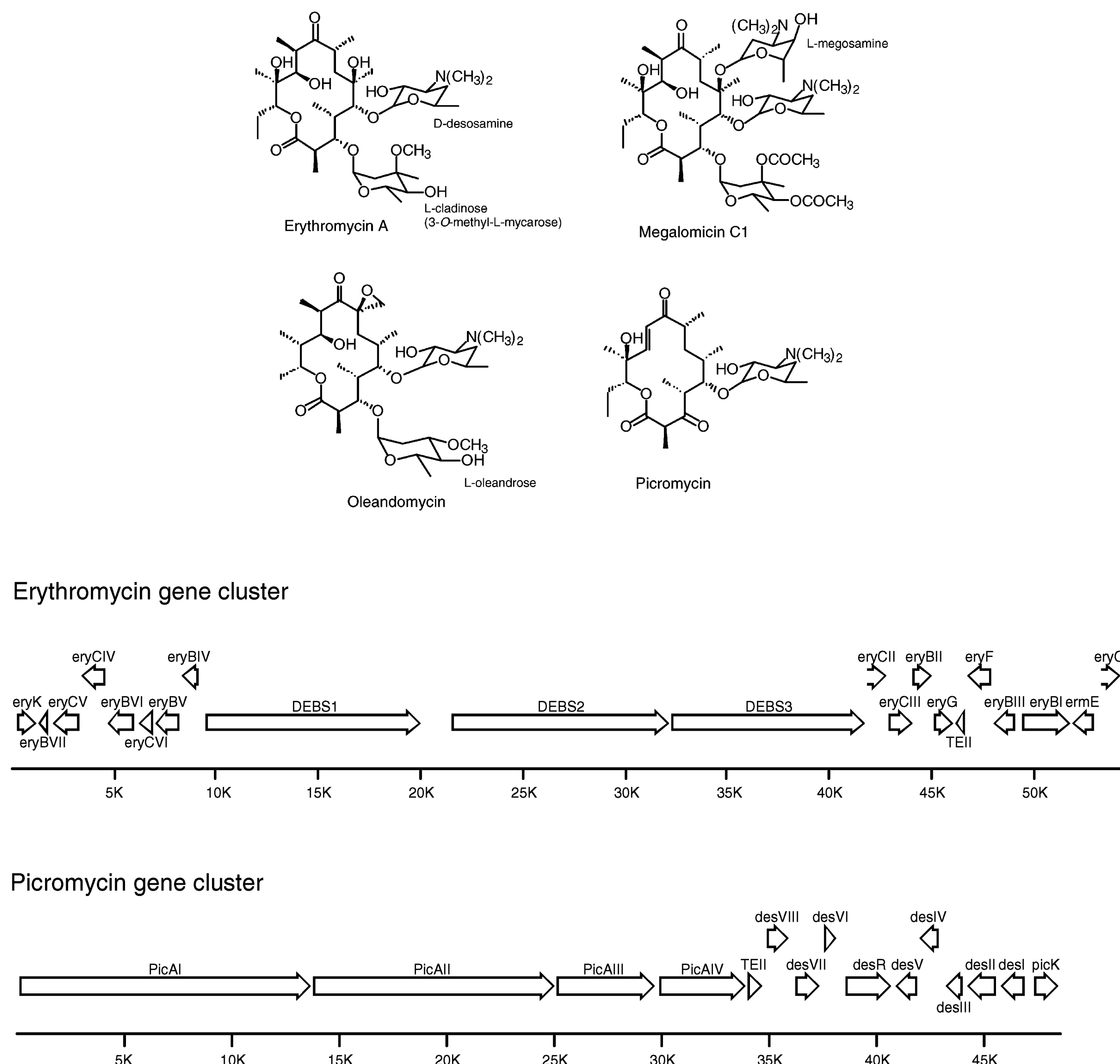


Figure 3. Fourteen-membered macrolide antibiotics and gene clusters.

samine formation (*megD*) and the *O*-acyl transferase (*megY*) have been identified in the *meg* cluster and expressed in the erythromycin-producing strain, *Saccharopolyspora erythraea*, to generate megalomicins in that strain.[35]

Oleandomycin contains an epoxide at C-8,C8a, introduced by OleP, a P-450 oxidase. The *oleP* gene has been coexpressed with DEBS to generate 8-hydroxy derivatives of 6dEB and 3-*O*-glycosylated 6dEB derivatives.[30,42] The two deoxysugars added to oleandolide are D-desosamine and L-oleandrose (3-*O*-methyl-L-olivose). The genes encoding TDP-L-olivose biosynthesis (*oleW*, *oleV*, *oleL*, *oleS*, *oleE*, and *oleU*) and its conversion to L-oleandrose (*oleY*) flank the OlePKS genes. These genes were used to generate 3-*O*-olivosyl and 3-*O*-oleandrosyl erythronolide B analogues[34] (see below). The genes encoding D-desosamine biosynthesis are also located within the *ole* cluster and homologous to those in the *ery*, *meg,* and *pic* clusters.

Picromycin and methymycin contain only the single deoxysugar, desosamine, encoded by the *des* genes. Gene knockouts of *des*VI,[43] encoding the *N*-methyltransferase, *des*V,[44] encoding a transaminase, and *des*I,[45] encoding a putative C-4 dehydrase, resulted in production of methymycin analogues with altered glycoside moieties. Attachment of desosamine to both the 12- and 14-membered lactones is performed by the same glycosyl transferase, encoded by *des*VII. After glycosylation, PicK (also called PicC), a P-450 oxidase, hydroxylates C-12 (picromycin) or C-10 (methymycin).[46,47]

3.2. Sixteen-Membered Macrolides

The 16-membered macrolide antibiotics characterized thus far fall into three different classes of polyketide backbones. These are represented by tylactone (**4**), platenolide (**5**), and mycinamicin lactone (**6**)/chalcolactone (**7**) (Figure 4), the polyketide components of tylosin, niddamycin/spiramycin, and mycinamicin/chalcomycin, respectively (Figure 5). The PKSs encoding each have been sequenced.[48−51] The organization of modules within subunits is conserved across all of the PKSs (Figure 4), comprised of the loading domain, module 1 and module 2 on the first subunit, module 3 on the second subunit, modules 4 and 5 on the third subunit, module 6 on the fourth subunit, and module 7 on the fifth subunit.

The chief difference among the 16-membered macrolide PKSs is the composition of extender units

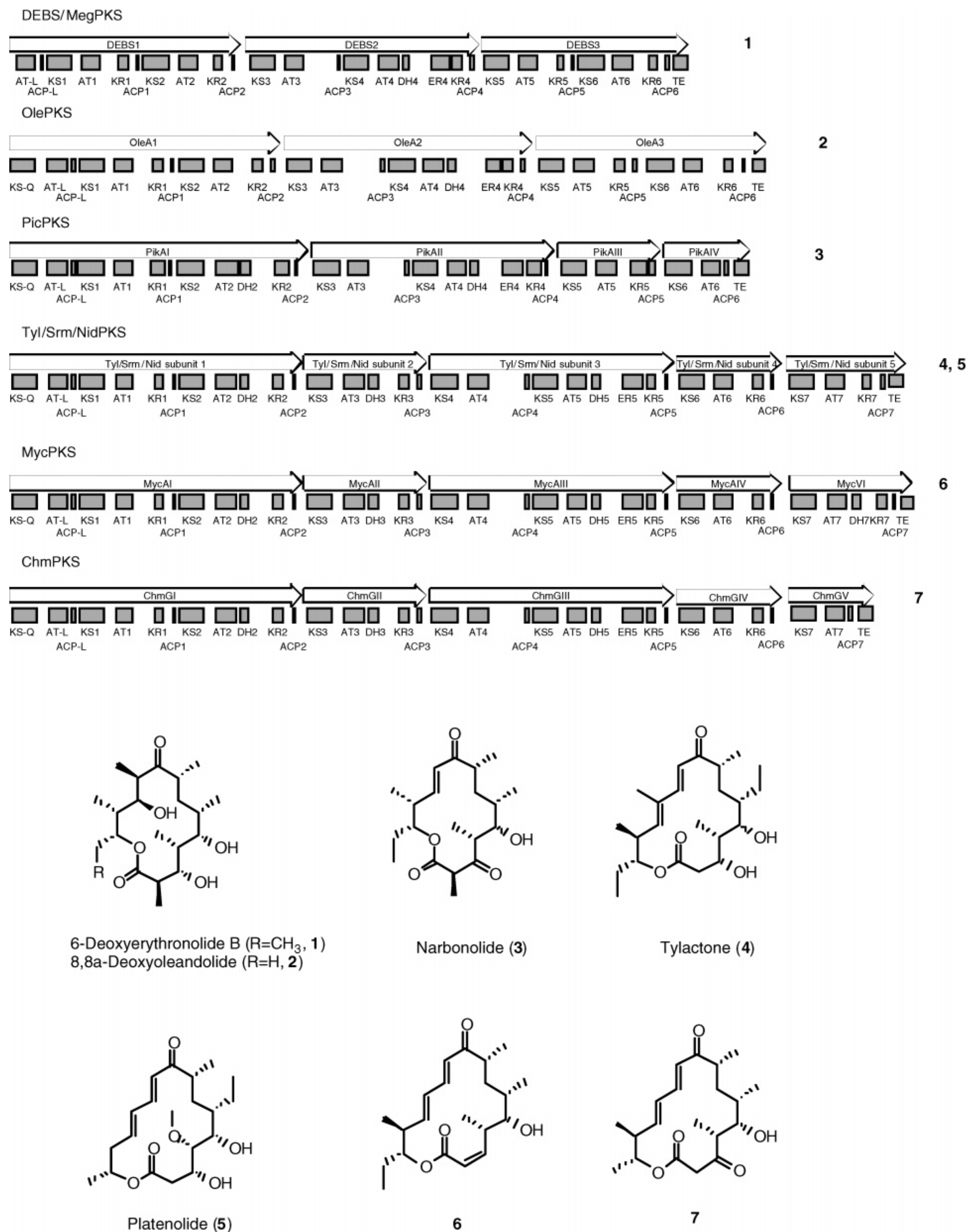


Figure 4. Organization of 14- and 16-membered macrolide PKSs and polyketide products.

utilized. Mycinamicin lactone and chalcolactone are derived exclusively from malonyl-CoA and methylmalonyl-CoA and differ only in the starter unit and C-3 functionality. Modules 5 of the tylactone and the platenolide PKSs utilize a 2-ethylmalonyl-CoA extender unit. A crotonyl-CoA reductase gene, *ccr*, is clustered with the tylosin biosynthetic genes (Figure 5) and presumably contributes to the flux of ethylmalonyl-CoA synthesis from four-carbon acyl-CoA pools during tylosin production.[50] Homologues of *ccr* are present in other PKS gene clusters requiring ethylmalonyl-CoA, and a homologue from *Streptomyces collinus* was expressed in *S. erythraea* containing a modified DEBS construct, which then incorporated ethylmalonyl-CoA at module 4.[52] In addition to the other three precursors, the platenolide PKS

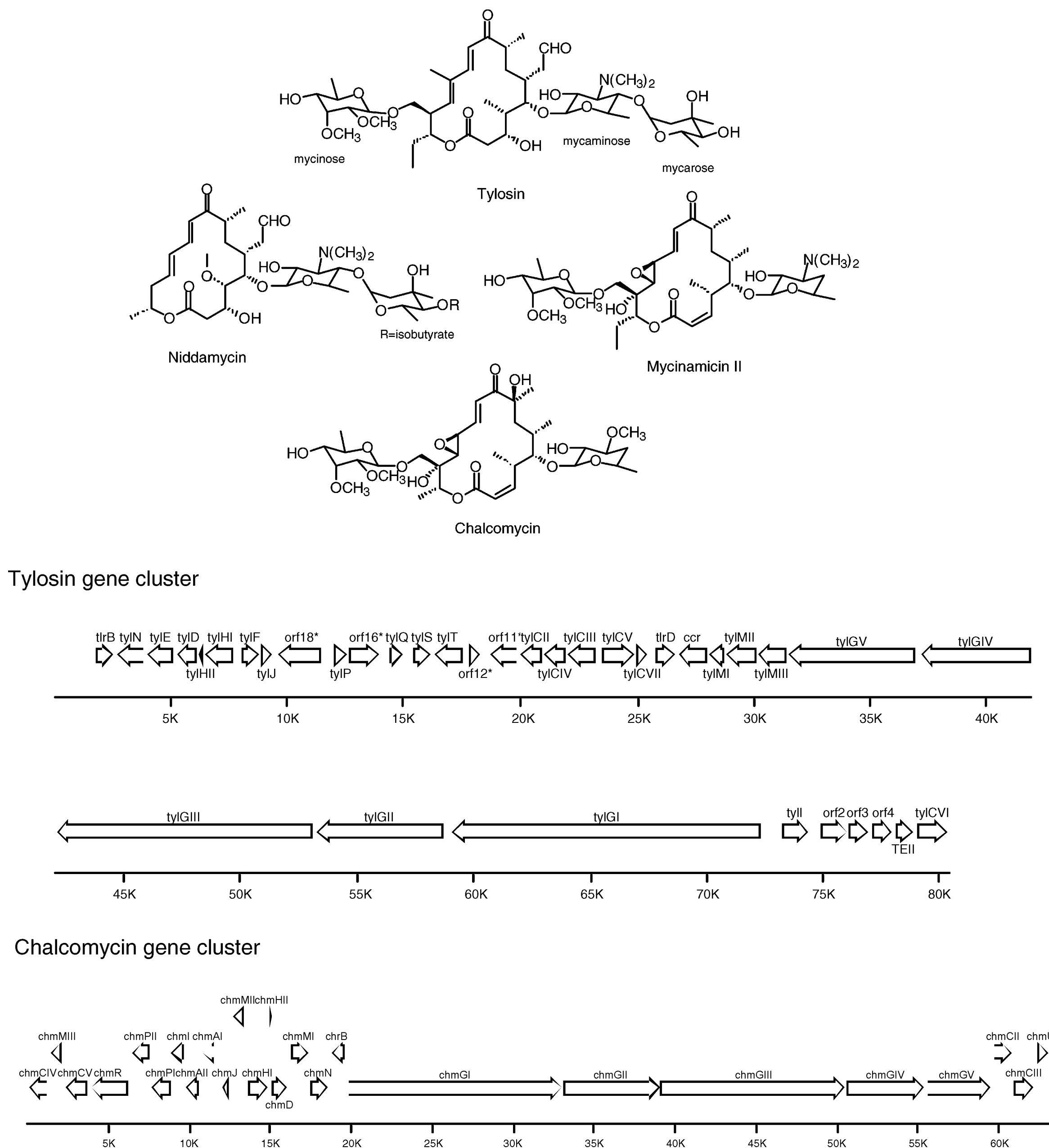


Figure 5. Sixteen-membered macrolide antibiotics and gene clusters.

also incorporates a 2-methoxymalonyl extender unit at module 6. Genes encoding the biosynthetic pathway of methoxymalonyl-ACP have been found in the FK520,[53] ansamitocin,[53] and geldanamycin gene clusters[54] (see below) and have been used to produce this precursor in other hosts.[55] Presumably, these sets of genes exist in the unsequenced regions of the 16-membered macrolide gene clusters that produce platenolide.

The genes for the biosynthetic pathways for all three deoxysugars attached to tylosin, D-mycaminose, L-mycarose, and D-mycinose (added as D-allose and modified to mycinose after attachment), have been sequenced from *S. fradiae*[56] (Figure 5). The *tylI* and *tylH* genes, both encoding P-450 oxidases, are responsible for the oxidations that occur on the C-6 ethyl and C-14 methyl branches of tylosin, respectively. Chalcomycin is one of the few macrolides that does not contain an amino-sugar at C-5 but rather has the neutral deoxysugar, D-chalcose, at that position (Figure 5). The putative genes for TDP-D-chalcose formation have been identified in the chalcomycin[51] and lankamycin[57] gene clusters. The genes for the remaining chalcomycin modifications, including TDP-mycinose biosynthesis/attachment and the two P-450s which hydroxylate C-8 and oxygenate C-12/C-13, have also been sequenced in the chalcomycin gene cluster.[51]

3.3. Ansamycins

The ansamycins are related to the macrolides biosynthetically but differ in the choice of starter unit (3-amino-5-hydroxybenzoic acid (AHBA)) and the lack of glycosylation. Formation of a macrolactam between the terminal carboxyl and 3-amino group of AHBA, instead of a macrolactone as above, results in a characteristic “basket with handle” molecular conformation.[58] Rifamycin, geldanamycin, herbimycin,

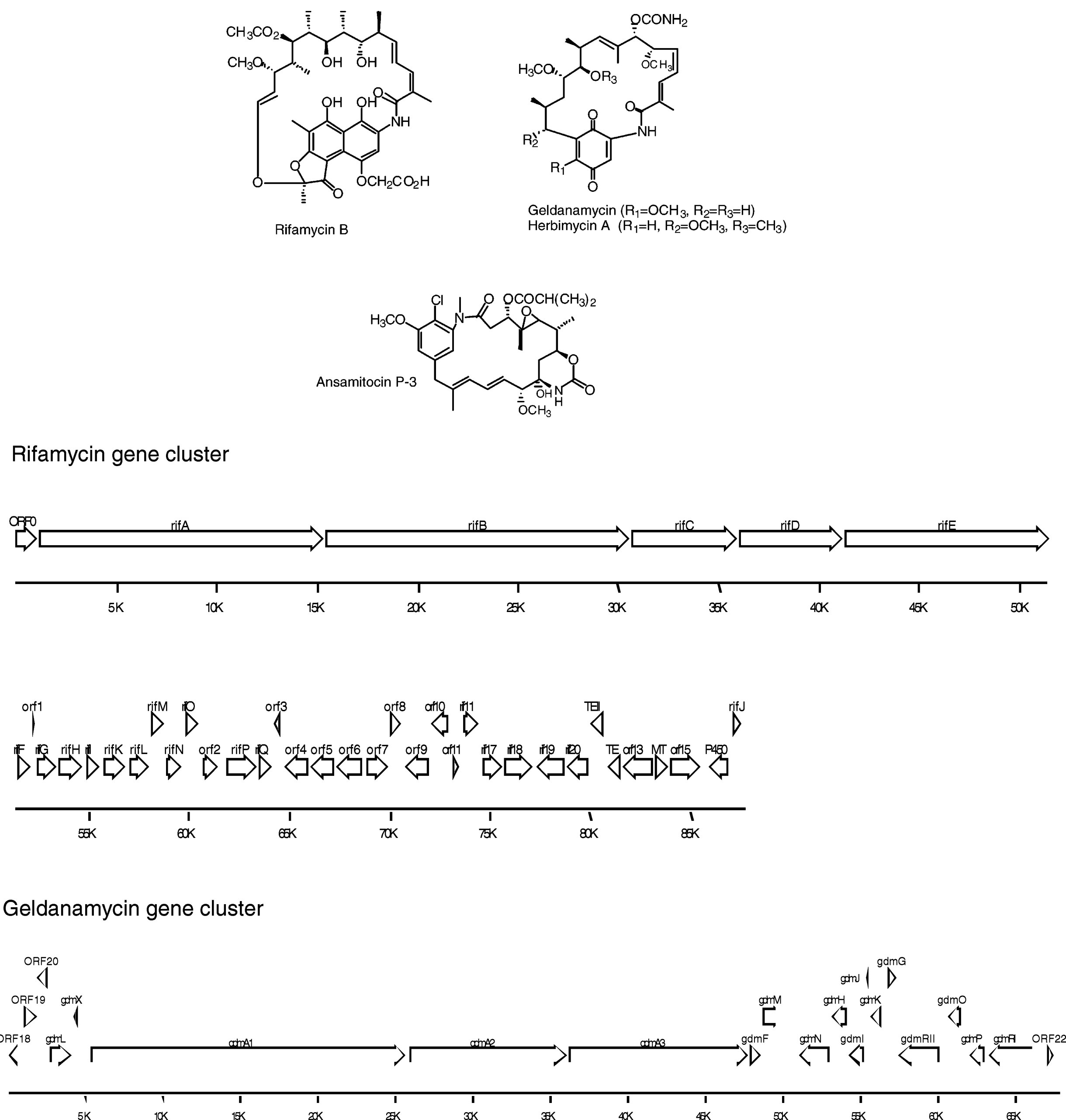


Figure 6. Ansamycin family of polyketides and gene clusters.

and the ansamitocins (Figure 6) are ansamycins for which the biosynthetic genes have been cloned and characterized. Derivatives of rifamycin are widely used in the treatment of tuberculosis, whereas geldanamycin analogues are undergoing clinical trials as antitumor drugs. The ansamitocins and closely related maytansine (found in plant extracts) are among the most cytotoxic polyketides known and are usually referred to as “maytansinoids”.[59]

Investigations of the rifamycin genes began with studies of the biosynthesis of AHBA at the enzymatic level, following the proof through isotopic labeling experiments that AHBA is the precursor of the studied ansamycins. The *rifK* gene was cloned by reverse genetics using information about the amino acid sequence of the AHBA synthase, RifK,[60] and then used to obtain the remainder of the AHBA biosynthesis, PKS and tailoring enzyme genes, by gene cloning and sequencing experiments.[61] Some of these genes were cloned independently by researchers at Novartis.[62] A notable feature of the modular rifamycin PKS (RifPKS) is its tendency to shed the enzyme-bound PKS intermediates spontaneously,[63,64] quite unlike the macrolide PKSs. This directly demonstrates the processivity of the RifPKS and shows that the 9,10-dihydronaphthoquinone ring of rifamycin is formed during this process to produce proansamycin X (**8**, Figure 7). Largely oxidative tailoring reactions remodel proansamycin X via rifamycin W (not shown) into the characteristic ansamycin framework of rifamycin B.[65] Initial attempts to modify the function of the RifPKS by the same type of domain inactivation experiments used for DEBS and other macrolactone PKSs (see below) have been greatly complicated by the spontaneous shedding of truncated PKS assembly intermediates (Y. Doi-Katayama, Y. J. Yoon, C. S. Park, and C. R. Hutchinson, unpublished work).

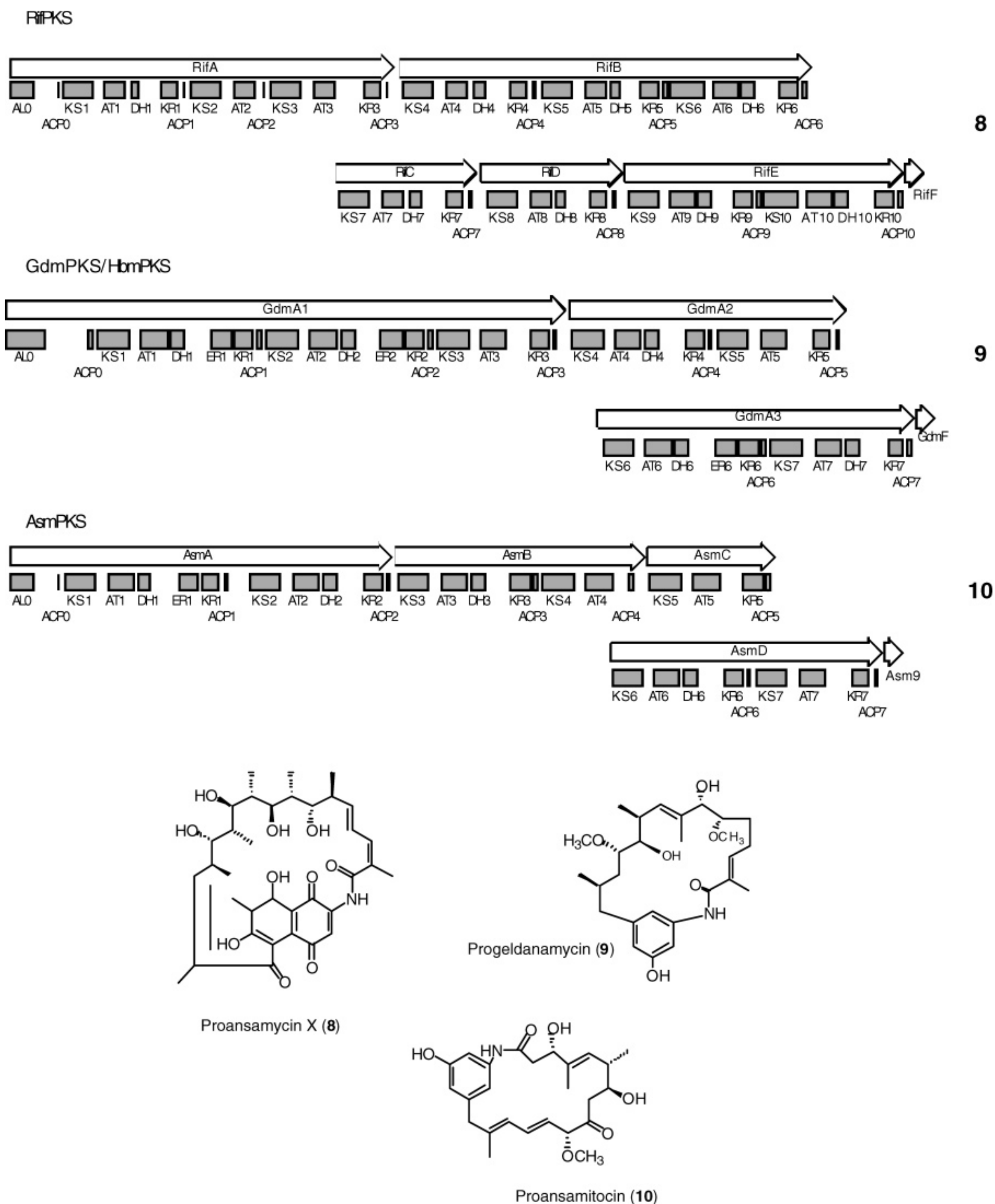


Figure 7. Organization of ansamycin PKSs and polyketide products.

In the ansamitocin gene cluster three of the AHBA biosynthesis genes are separated from the rest of the *asm* genes by at least 30 kb of intervening DNA.[59,66] Disruption of many of the tailoring enzyme genes has been used to develop a picture of the sequence of biosynthetic steps,[67] and the substrate specificity of the Asm19 *O*-acyltransferase has been defined through studies of the purified enzyme.[68] This enzyme distinguishes the ansamitocins (3-*O*-acylesters) from maytansine (3-*O*-(*N*-acetyl-*N*-methyl-L-alanine ester). As noted above, the *asm* cluster also contains a set of five genes for formation of 2-methoxymalonate, one of the substrates used by the *asm* PKS for chain elongation. In fact, studies of these genes along with their orthologs from the FK520-producing streptomycete have elucidated what is known about the biosynthesis of this atypical substrate.[55,69]

Geldanamycin and herbimycin are made by distinct *Streptomyces hygroscopicus* strains, but their biosynthesis and gene clusters are nearly identical. Rascher et al.[54] described a major portion of the geldanamycin genes, which, like those of ansamitocin biosynthesis, occur as two separate clusters. One cluster (Figure 6) contains the PKS and tailoring enzyme genes plus one AHBA biosynthesis gene, *gdmO*; the rest of the AHBA biosynthesis genes lie somewhere else in the chromosome yet are essential for geldanamycin biosynthesis, but not primary me-

tabolism, because their disruption abolishes geldanamycin production (A. Rascher and C. R. Hutchinson, unpublished results). The corresponding herbimycin PKS gene cluster is nearly identical, lacking only the *gdmF* (amide synthase) and *gdmM* (monooxygenase) orthologs (A. Rascher, Z. Hu, and C. R. Hutchinson, unpublished results). Of the *gdm* tailoring genes, *gdmM* and *gdmN* have been characterized functionally by gene knockout experiments[54] (A. Rascher and C. R. Hutchinson, unpublished results). Extensive engineering of the geldanamycin PKS (GdmPKS) genes (Figure 7), aided by development of convenient methods for their modification and expression from integrative vectors, has provided several geldanamycin analogues.[70] Some of these are undergoing evaluation as antitumor drugs at Kosan Biosciences in the quest for analogues with reduced hepatotoxicity and greater water solubility.

4. Overview of Methodologies

4.1. Modular PKSs

A number of strategies for reprogramming modular PKSs at the genetic level have emerged over the past decade, ranging from single point mutations to multiple module replacements, all resulting in polyketides with targeted structural modifications. Most of these strategies have been discussed in previous reviews,[4−6,8,9,11,18,20] and an outline is presented here. Briefly, these strategies include active site inactivation or replacement, module substitution, subunit complementation, and precursor-directed biosynthesis.

The most common method of engineering thus far is AT substitution, in which the native AT domain is replaced with an AT encoding a different starter or extender unit specificity. The AT used for substitution is usually derived from a heterologous modular PKS. All six of the methyl-malonyl-specific AT domains in DEBS have been successfully replaced by malonyl-specific AT domains to create 6dEB or erythromycin analogues lacking a corresponding methyl branch.[4,71−73] Similar replacements have also been accomplished with the spiramycin,[50] FK520,[74] rapamycin (J. Kennedy, personal communication), and geldanamycin PKSs (unpublished data). The loading AT of DEBS has been replaced by loading ATs or AT/KS^q domains from the avermectin,[75,76] tylosin, and oleandomycin and rapamycin PKSs[77] for production of C-13-substituted derivates of erythromycin. Replacing an AT domain in DEBS with an ethylmalonyl-specific AT[52] and a 2-methoxymalonyl-specific AT[55] domain to generate ethyl and methoxy branches, respectively, in either erythromycin or 6dEB has also been performed successfully. In each case, introduction of exogenous genes involved in precursor metabolism (ethylmalonyl-CoA and 2-methoxymalonyl-ACP) from other PKS gene clusters was required for production in the engineered host.

Manipulation of β-keto processing activities has been accomplished by inactivation of domains, deletion of domains, and substitution of domains. Nearly all of these examples have been performed with DEBS. Examples of inactivation include the ER domain in module 4 by site-directed mutagenesis to generate a Δ6,7-anhydro erythromycin derivative[78] and deletion of KR domains in modules 5 and 6 to generate C-3 and C-5 keto derivatives of 6-dEB, respectively,[15,79] and replacement of the KR domains in modules 2, 5, and 6 to produce C10−11, C4−5, and C-2−3 anhydro derivatives of 6dEB.[79] Similarly, substitution of KR domains in modules 2 and 6 with a DH+ER+KR domain resulted in C-10 and C-3 deoxy 6dEB analogues.[79]

There is at least one report of a single whole module substitution, DEBS module 2 with rifamycin module 5.[80] Although the activities of the two modules are identical and production of 6dEB was reproduced, it suggests a useful alternative route in situations where standard domain engineering falls short. Construction of hybrid modules, subunits, and subunit complementation within families of related PKSs has been demonstrated in a number of cases. Examples include DEBS/PicPKS,[30,81] DEBS/rapamycin PKS,[10,82] DEBS/OlePKS,[41] PicPKS/OlePKS,[83] PicPKS/TylPKS,[81] and chalcomycin PKS/spiramycin PKS.[84] In many of these experiments the production levels of the polyketide remain relatively high, demonstrating effective communication between noncognate subunits.

Precursor-directed biosynthesis combines the advantages of modern synthetic chemistry with complex polyketide biochemistry to create a powerful approach to engineering polyketide chains with unique features. This is achieved by constructing strains which are blocked at a particular step in the pathway (e.g., a KS domain) or in the ability to produce a necessary precursor and supplying synthetically derived precursors in the form of *N*-acylcysteamine thioesters.[85,86] Several novel 14- and 16-membered lactones have been produced using a KS1° derivative of DEBS and supplying synthetic diketide or triketide precursors.[87−89] Side chains harboring halogens and reactive groups for further modification by synthetic chemistry have been incorporated into 6dEB. To date, this has been the most successful approach to making macrolides with potency equal to or better than erythromycin.[6]

4.2. Aromatic PKSs

Although the field of polyketide gene engineering was catalyzed by work with aromatic PKSs, this class has not been exploited as intensely as modular PKSs. Most successful attempts at engineering aromatic polyketides result from "mixing and matching" of separate individual enzyme subunits. For example, pairs of ketosynthase and chain length factors (KS/CLF), which together synthesize a length-specific polyketide backbone, ketoreductases, cyclases, and aromatases from different aromatic gene clusters have been combined to manipulate chain length, hydroxylation pattern, cyclization regiospecificity, and aromaticity.[90−95] A recent advance is the ability to manipulate the choice of starter unit incorporation through the use of a separate initiation module, following identification of such a unit by the study of the gene clusters for some of the rare aromatic polyketides that use nonacetate starters.[96] Cycliza-

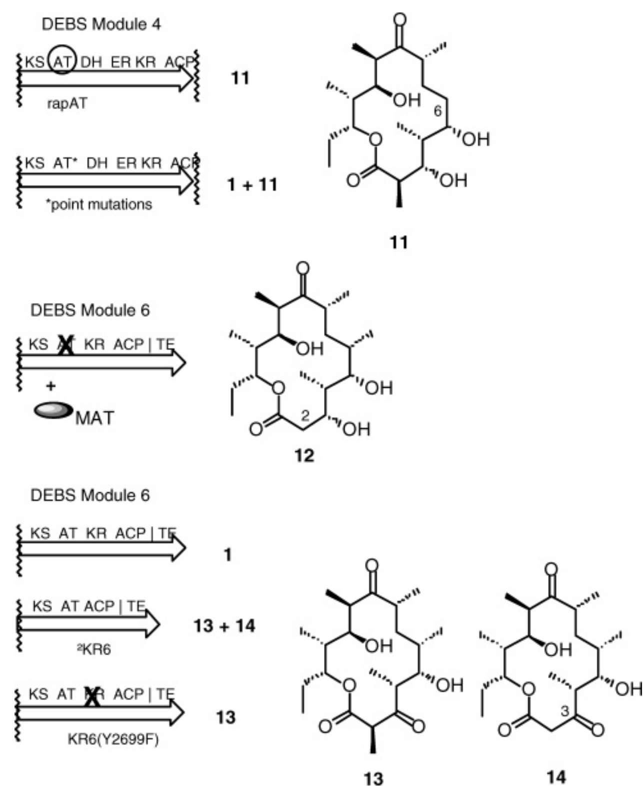


Figure 8. Examples of recent strategies for AT and KR engineering. See sections 5.1.1 and 5.1.2 for details.

tion of the polyketide chain can be controlled with appropriate choice of cyclases and aromatization enzymes, provided one can be found with the desired specificity. In the absence of such enzymes, however, the final product structure(s) is determined by spontaneous cyclization, which may be difficult to predict. Post-PKS steps such as oxidation, *O*-methylation, or glycosylation are more amenable to alteration, as illustrated by the work of Salas and co-workers with mithramycin and related chromanequinone antibiotics.[97−100]

5. New Developments in Modular PKS Manipulation

5.1. Modeling and Engineering of PKS Domains

5.1.1. Acyltransferases

Not every attempt at modular PKS AT domain replacement is successful when a conserved set of boundaries is used across different PKS modules. At least one major reason for such unproductive AT swaps is an apparent disruption in protein structure so that chain elongation catalyzed by the KS and ACP domains is severely attenuated.[101] Recent experiments suggest that the choice of the domain boundaries used to create the hybrid enzyme can be a critical determinant of success. For example, our group failed to engineer a productive malonyl-AT domain replacement in module 4 of DEBS using several different domain junctions.[102] However, a set of alternative junctions used by Leadlay and co-workers did result in a productive AT replacement[73] (Figure 8).

Two alternative approaches to wholesale AT swaps recently described are site-directed mutagenesis to alter the specificity of an AT domain[102,103] and inactivation of an AT, followed by complemention with a separate trans-acting AT subunit[104] (Figure 8). Both methods hold the advantage that structural modification to a domain is minimized and potentially detrimental perturbations are avoided. In the former case, the crystal structure of the *E. coli* fatty acid malonyl transferase and sequence alignments were used to identify residues putatively involved in substrate specificity. These mutations were introduced into the AT4 domain of DEBS to produce 6-desmethyl-6dEB (**11**),[102] although the mutations led to relaxed specificity—6dEB (**1**) was also produced—rather than a complete change in specificity. Actual structures of modular PKS AT domains may help refine the residues that are involved in substrate selection and permit engineering of ATs with more stringent specificity. In the second example, an AT6-null mutant of DEBS was created by mutation of the active site and the PKS complemented with a type II fatty acid malonyltransferase (MAT) from *S. coelicolor* to produce 2-desmethyl-6dEB (**12**) in *E. coli* with yields similar to that of the wild-type PKS.[104]

5.1.2. Ketoreductases

KR domains in modular PKSs catalyze stereospecific reduction and may be classified into two groups according to the stereochemical outcome relative to the polyketide backbone. Many examples of both types of KRs exist. Inversion of an alcohol stereocenter is possible by replacing a KR of one class with a KR from the other,[79,105] but relatively few examples have been reported, and all were performed at the terminal module. Alteration of KR specificity in a module preceding another module may require concomitant modification of the downstream KS so that the altered stereocenter is recognized and processed.

Recent studies with sequence alignments of the two KR types have identified differences in amino acid residues that correlate with stereospecificity.[106,107] The perfect correlation of these residues allows one to predict stereochemical outcome in cases where a gene sequence is known but the final product or absolute stereochemistry is not known. Two models of substrate binding relative to the NADPH cofactor have been proposed to explain the relative outcomes of ketoreduction,[106,108] and Caffrey[107] has proposed mechanisms by which these residues may dictate specificity in either model.

Homology modeling to the short-chain dehydrogenase/reductase family suggested a putative catalytic triad in modular KRs.[106] Point mutation of the catalytic serine resulted in complete inactivation of the KR6 domain in DEBS, producing 3-deoxy-3-oxo-6dEB (**13**). Modification of the KR by this method resulted in only the targeted analogue, whereas deletion of the entire KR6 domain in DEBS affected the specificity of the adjacent AT domain and led to unexpected products (**14**) as well (Figure 8). This particular example stresses the benefit of engineering strategies which seek to minimize structural disturbances. The same mutation has been used to inactivate KR domains in the epothilone[109] and geldana-

mycin PKSs (manuscript submitted) and produce corresponding analogues.

5.2. Type II Thioesterase

Modular PKS gene clusters commonly contain genes encoding a type II thioesterase (TEII), defined initially through studies in *E. coli* as hydrolytic enzymes acting on long-chain fatty acid thioesters.[110] These enzymes are not involved in the terminal event of PKS assembly like the thioester domains discussed below and, to some degree, are dispensable because inactivation of their genes does not always abolish polyketide formation, although product titers usually are drastically lowered, as in the case of tylosin[111] and picromycin.[36] In erythromycin biosynthesis by *S. erythraea*, genetic and biochemical studies have shown that the *ery*-ORF5 TEII enzyme favors hydrolysis of acetyl groups bound to the loading ACP domain to ensure formation of 6dEB from a propionate instead of an acetate starter unit.[112] Thus, the most likely role of such enzymes is to edit the process of PKS assembly by selective hydrolysis of misprimed or -acylated active site cysteines, as directly shown by Schwarzer et al.[113] in the case of TEII enzymes of nonribosomal peptide antibiotic biosynthesis. Understanding the exact contributions made by TEIIs will be critical for optimizing productivity of engineered modular PKSs.

5.3. Cyclization/Termination

The TE domains attached to the terminal modules of PKSs are generally tolerant toward polyketide chain length as well as substitutions at the C-2 and C-3 positions of the lactone, although with varying efficiencies. The TE domains from DEBS and PicPKS have been studied in vitro[89,114] and can cyclize lactone ring sizes in the range of 6−16 carbons. The PicPKS TE has a much higher preference for 3-keto versus 3-hydroxy substrates as compared to DEBS TE, and fusion of the PicPKS TE to DEBS module 3 resulted in an enzyme with greater efficiency than DEBS module 3 with DEBS TE, indicating that the PicPKS TE is a better catalyst for cyclizing 3-keto acyl intermediates.[114] Crystal structures of both TE domains from DEBS[115] and PicPKS[116] have now been obtained and, in addition to providing clues about the overall tertiary architecture of modular PKSs, provide a structural basis for potentially altering substrate specificity. In addition to cyclizing lactones from a variety of macrolide PKSs, DEBS TE was used recently to generate a novel pentaketide lactone from the spinosyn PKS.[117]

The ansamycin PKSs, unlike macrolide PKSs, utilize a separate protein for cyclization of the linear polyketide to the corresponding macrolactam. These amide synthases, RifF (rifamycin), GdmF (geldanamycin), and Asm9 (ansamitocin), are homologous to *N*-acetyl CoA transferases. The degree of substrate flexibility among this class of enzymes is not currently known but if similar to the TEs could be useful for engineering novel lactams.

5.4. Intermodular and Intramodular Communication

Accurate programmed polyketide extension depends on several protein−protein interactions to correctly orient catalytic domains relative to ACP-linked substrates as well as facilitate specific intermodular transfer of the growing polyketide chain. In addition to such structural communication, proper extension requires compatibility of enzyme specificities such that each intermediate is accepted through the reaction sequence. Our ability to reorganize PKS structure for the synthesis of novel compounds will depend on our understanding the rules for functional inter- and intramodular communication and the flexibility with which we can modify communication independent of catalytic activity.

Recent work suggests that one reason domain exchange has generally yielded poorly active PKSs could be extra-domain intramodular structural perturbation. An engineered DEBS module 6+TE, in which the AT was replaced with that from RAPS module 2, was shown to retain nonlimiting malonyl transferase and KS loading activities but was impaired in substrate condensation.[101] These results suggest that the AT insertion may disrupt correct interaction between ACP and KS domains necessary for the C−C bond-forming extension reaction.

Much work of late has focused on the determinants of intermodular interaction that facilitate the specific channeling of the growing polyketide chain. There is clear evidence that a significant role is played by the intervening protein sequence between the ACP domain and the KS of the downstream module[80,104,118−122] (Figure 9). These sequences, which have been termed intermodular “linkers”, facilitate both intra- and interpolypeptide substrate channeling. Intrapolypeptide linkers bridge modules within the same protein. Interpolypeptide linkers are terminal sequences that bridge C- and N-terminal modules on separate proteins.

There is considerable evidence that module−module interaction can be engineered independently of module catalytic function. Linkers can be altered or exchanged without loss of intrinsic module activity, and several hybrid PKSs have been successfully constructed using compatible interpolypeptide linkers to bridge normally incommunicative modules.[80,119−122] Thus, engineering of linkers may allow flexibility in the physical reorganization of multimodular PKSs.

Recent evidence suggests that acyl chain substrate specificity can be a significant barrier to intermodular channeling. Specificity in the transfer of the acyl chain from the upstream ACP to the next module KS, in the KS-catalyzed condensation reaction, or in eventual product release by a TE domain may restrict elongation of novel compounds on hybrid PKSs. A thorough study of the substrate selectivities of several PKS modules has given interesting insights into some specificity determinants.[123] In particular, it was observed that modules unable to extend substrates were blocked not at acyl chain transfer to the KS but at subsequent condensation, implying that somehow transition-state stabilization in the rate-limiting condensation reaction is more stringent than that in

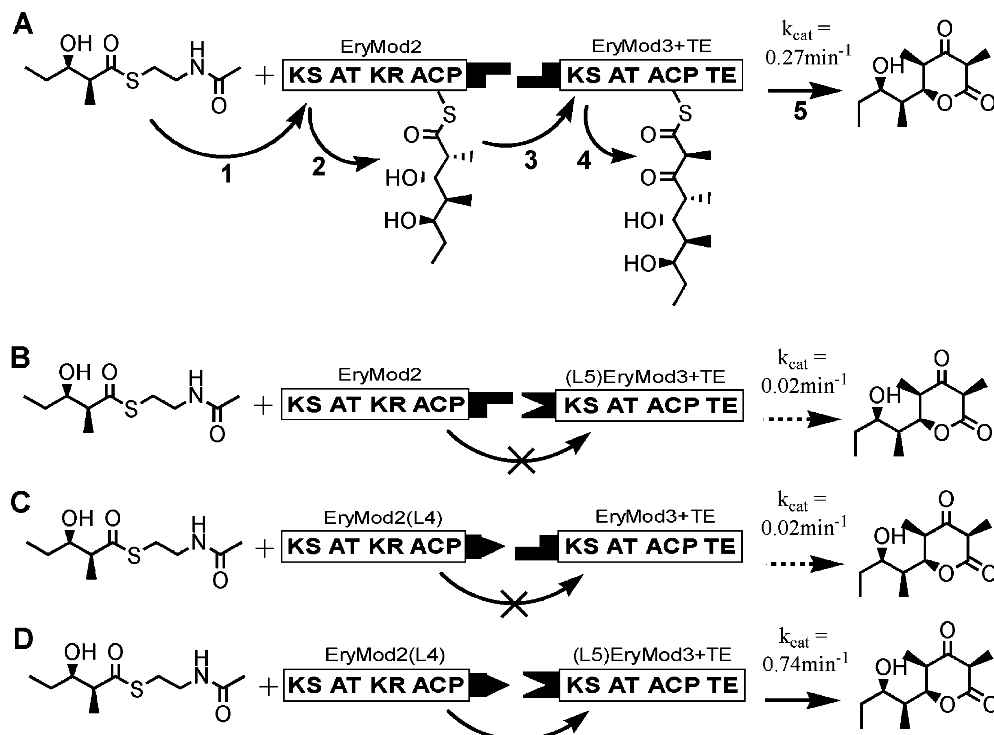


Figure 9. Intra- and intermodular communication in polyketide extension. (A) Some interactions critical to substrate channeling are shown: 1 and 3, substrate acceptance by KS; 2 and 4, condensation of KS- and ACP-bound intermediates; 5, product hydrolysis by TE. Intermodular transfer via specific interpolypeptide linkers is illustrated in a comparison of panels A–D. Matched linker pairs (A and D) from DEBS modules 2 and 3 (L2 and L3) or modules 4 and 5 (L4 and L5) greatly facilitate intermodular transfer of the growing polyketide chain. Data shown are taken from Tsuji et al.[119]

acyl transfer. Understanding the basis for this discrimination may allow us to change or broaden specificity, opening new routes to functional hybrid PKSs and their diverse potential products.

5.5. Precursor Engineering

PKSs occasionally require substrates for polyketide biosynthesis that are not commonly found in bacterial cells, for instance, 2-methoxymalonate and AHBA. Unlike the ubiquitous small branched-chain fatty acids used as starter units, which are believed to come from the catabolism of valine, leucine, and isoleucine,[124] formation of 2-methoxymalonate for the biosynthesis of FK520, ansamitocins, and geldanamycin requires a dedicated set of five genes to convert some glycolytic intermediate to the ACP-bound form of 2-methoxymalonate (**15**), as currently believed (Figure 10). The acyl ACP dehydrogenase gene product has been studied in vitro,[125] and heterologous expression of the genes from the ansamitocin and FK520 producers in *S. coelicolor* or *S. fradiae* has been used to produce, respectively, an erythromycin[55] and a midecamycin[126] analogue.

E. coli is being developed as a host for polyketide production,[127,128] which also requires the introduction of metabolic pathways to make PKS substrates. Pathways to form (2*S*)-methylmalonyl-CoA (**16**) from propionate via propionyl-CoA carboxylase[128] or from succinyl-CoA via methylmalonyl-CoA mutase[129] have been engineered to support polyketide production of 6dEB (**1**) and analogues in *E. coli* (Figure 10). A 15-methyl-6dEB analogue was produced in *E. coli* by overexpressing the acetoacetyl-CoA:acetyl-CoA trans-

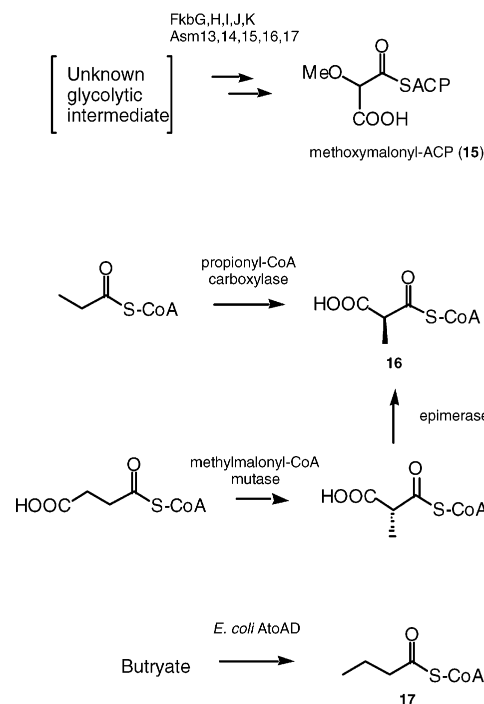


Figure 10. Precursor pathways that have been engineered in *S. coelicolor* and *S. fradiae* (**15**) and *E. coli* (**16** and **17**).

ferase (*atoAD*) to generate butyryl-CoA (**17**), which is utilized by the loading AT of DEBS, from fed butyric acid or butanol.[130]

5.6. Tailoring Pathways

Desosamine is present on all of the 14-membered macrolides and is essential for antibiotic activity.

Recent crystallography studies with erythromycin bound to ribosomes reveal the essential role that desosamine plays in binding to the ribosome.[131] Fourteen-membered lactones lacking desosamine are completely inactive, and no synthetic substitute or modification to desosamine has yet been found which maintains the same level of activity. The entire set of *des* genes, including the glycosyl transferase from the picromycin cluster, were coexpressed with modified DEBS genes which produce analogues of 6dEB to produce a small library of 14-membered macrolides containing only desosamine.[132] Antibiotic activity was detected in nearly every case, suggesting desosamine is sufficient to confer antibiotic activity to macrolactones.

Several experiments have been conducted to introduce modified or unnatural deoxysugars to macrolide backbones (reviewed in Mendez and Salas[133]). Two approaches to producing desosaminylated tylosin derivatives have been described. In one example, the TylMII mycaminosyltransferase was expressed in an engineered *S. erythraea* strain which does not make erythromycin and used to bioconvert tylactone to 5-*O*-desosaminyl-tylactone.[134] In the other case, two genes involved in desosamine biosynthesis from narbomycin were imported into the tylosin producing strain, *Streptomyces fradiae*, to convert TDP-D-mycaminose (TDP-D-4-deoxydesosamine) to TDP-D-desosamine and produce desosaminylated tylonolide derivatives.[135] The genes encoding L-olivose and L-oleandrose biosynthesis were expressed from plasmids in *Streptomyces albus* and used to bioconvert erythronolide B to 3-*O*-olivosyl and 3-*O*-oleandrosyl erythronolide B analogues.[136] These and other experiments indicate some degree of deoxysugar substrate flexibility and the ability to engineer novel glycosides of macrolide antibiotics. However, to date, such modifications have not led to compounds with activity superior to the natural glycosides.

6. Creating and Improving Microbial Production Systems

In addition to genetically engineering PKSs and manipulating tailoring enzymes, another genetic approach to novel metabolites has involved the development of heterologous bacterial hosts. This work has had two goals: to expedite structure–function investigations of PKSs and the exploration of metabolic pathways and to improve polyketide titers by faster means than lengthy strain improvement carried out by random mutation and screening.

E. coli is becoming a versatile host for the expression of modular PKS genes, as demonstrated by the recent work at Stanford University and Kosan. Pfeiffer et al.[128] produced 6dEB and novel analogues made from aromatic acid starter units, and Kosan scientists made a wide range of 6dEB analogues with novel substituents at C-13 derived from either butyric acid[130] or fed *N*-acetylcysteamine thioesters (J. Kennedy et al., manuscript in preparation). Recent work with the rifamycin[137,138] and epothilone PKS genes[139] (S. Mutka et al., submitted for publication) and certain deoxysugar biosynthesis genes (H. Gramajo et al., manuscript in preparation) have shown that *E. coli* is likely to be a quite versatile host for polyketide pathway engineering.

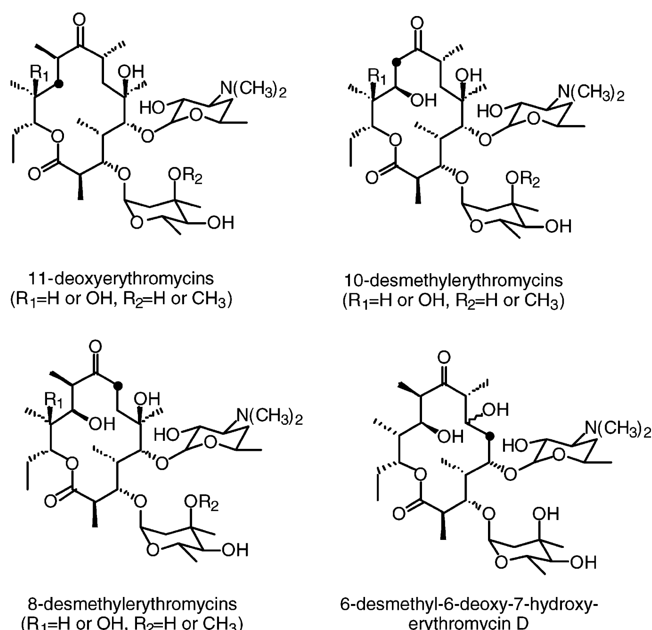


Figure 11. Erythromycin analogues produced by engineered DEBS genes expressed in an overproducing *S. erythraea* host.

Another useful approach is to use an industrial strain whose titer has already been improved as the host for expression of PKS genes to gain the benefit of the presumably enhanced levels of gene expression, substrates, or tailoring enzymes that could be present. Two examples of this strategy have been described in which the native PKS genes were deleted from the high-producing strain and replaced with the genes from a wild-type producer. In both cases, use of the wild-type genes did not lower the polyketide titer, demonstrating that increased titers in the industrial strains were not due to PKS mutations. Engineered DEBS genes were then expressed in the high-producing *S. erythraea* strain to generate erythromycin analogues at substantially higher levels than those obtained in *S. coelicolor*[140,141] or the wild-type *S. erythraea* strain (Figure 11). More recently, a high-producing *S. fradiae* strain has been used to produce a number of novel 16-membered macrolides in high yield.[84,126]

7. Conclusions

Since the initial cloning of genes for polyketide biosynthesis in the late 1980s and early 1990s, a number of strategies and tools for engineering the genes and pathways to create new polyketide compounds have been developed. These have been used to create analogues of structurally complex compounds that would be difficult to obtain through conventional organic synthesis or semi-synthesis. Though many of the strategies are empirically based, they are fairly robust when compared to the success rate of a typical synthesis reaction as applied to different substrates and so can be viewed as a practical and complementary tool in the design and production of new therapeutic entities. These techniques should be refined through continued practice,

and additional methods will likely emerge to provide increased versatility. Current and future information derived from ongoing biochemical and structural studies of PKSs will certainly help guide these approaches.

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