8
Recent Developments in the Production of Novel Polyketides by Combinatorial Biosynthesis

T. ASHTON CROPP[1], BEOM S. KIM[1], BRIAN J. BECK[2], YEO JOON YOON[2], DAVID H. SHERMAN[2] AND KEVIN A. REYNOLDS[1*]

[1]*Department of Medicinal Chemistry and Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, Virginia 23219, USA and* [2]*Department of Microbiology, University of Minnesota, MMC 196, 420 Delaware Street, S.E., Minneapolis, MN 55455, USA*

Introduction

Polyketides are a class of structurally diverse natural products which possess a wide range of biological activities (Hopwood, 1997). These compounds are used throughout medicine and agriculture as antimicrobials, immunosuppressants, antiparasitics, and anticancer agents.

While structurally diverse, polyketides are assembled by a common mechanism of decarboxylative condensations of simple malonate derivatives by polyketide synthases (PKSs) in a manner very similar to fatty acid biosynthesis. After assembly by the PKS, tailoring enzymes such as glycosyltransferases, hydroxylases, or methyltransferases can then further modify the polyketide product. These post-PKS modifications are almost always necessary in order for the molecule to be bioactive. It has been shown that, frequently, bacteria (primarily the actinomycetes) possess both multifunctional (type I) and multicomponent (type II) PKSs. The first type I PKS to be sequenced and characterized was 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea*, which produces the polyketide macrolactone ring of erythromycin (*Figure 8.1*) (Cortes *et al.*, 1990; Donadio *et al.*, 1991; Caffrey *et al.*, 1992). This modular PKS contains a loading module responsible for selecting a primer unit (in this case, propionyl CoA) and six extension modules. It was shown

* To whom correspondence may be addressed (kareynol@hsc.vcu.edu)

Abbreviations: PKS, polyketide synthase; CoA, co-enzyme A; AT, acyltransferase; KS, β-ketosynthase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase; ER, enoyl reductase; TE, thioesterase; DEBS, 6-deoxyerythronolide B synthase.

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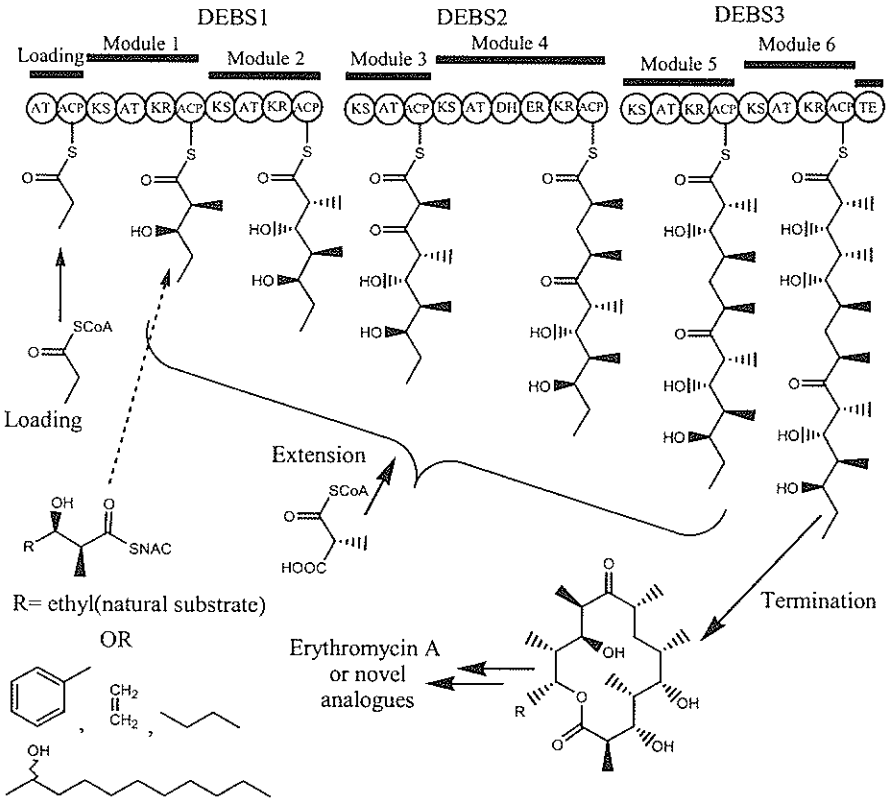

Figure 8.1. Organization of the 6-deoxyerythronolide B synthase (DEBS). Inactivation of KS1 allows for the ability to feed diketide *N*-acetylcysteamine (NAC) thioesters, which are loaded and extended to yield novel erythromycins.

that each PKS extension module contains an acyltransferase domain (AT), which loads an acyl carrier protein (ACP) with the appropriate extender unit. In the case of DEBS, the AT domains have substrate specificity for methylmalonyl CoA extender units, but other systems are capable of incorporating malonyl or ethylmalonyl CoA as well. The activated ACP is then condensed with the growing polyketide chain by a ketosynthase domain (KS). In addition, ketoreductase (KR), dehydratase (DH), or enoyl reductase (ER) domains may be present to further modify the β-keto group of the polyketide chain. The last module contains a C-terminal thioesterase domain (TE) responsible for cyclization of the macrolactone. This initial research on DEBS has spurred the discovery of other PKS systems (Aparicio *et al.*, 1996; August *et al.*, 1998; Xue *et al.*, 1998; Ikeda *et al.*, 1999; Molnar *et al.*, 2000) which, although producing a variety of different products, have the same modular arrangement as DEBS.

Hybrid PKSs

The same structural diversity that makes the polyketides such a useful class of natural products also limits the development of novel analogues. The complexity involved almost always prevents total synthesis for large-scale preparation of a compound and has, historically, limited the number of polyketides to the natural metabolites or simple, semi-synthetic derivatives. Recent advances in molecular genetics, however, have opened the door to engineering novel secondary metabolites in a variety of organisms. The modular architecture of PKSs allows for the possibility of combining or altering individual domains or modules in an effort to produce novel polyketides, a process known as combinatorial biosynthesis (Katz, 1997; Khosla, 1997). Using DEBS as a scaffold, successful examples of hybrid PKSs have been constructed and shown to produce 'unnatural' polyketides. These combinations have been made using individual catalytic domains (Ruan *et al.*, 1997; McDaniel *et al.*, 1999), whole modules (Marsden *et al.*, 1998), or entire subunits (Tang *et al.*, 2000). This exciting work has generated a number of new polyketide analogues, which would be very difficult to obtain synthetically. Unfortunately, the yields of these novel polyketides are frequently lower than the natural compound for reasons that are not entirely clear. The fact that new products are made, however, indicates hybrid PKSs can work and that there is significant flexibility in the PKS complex and enzymatic domain active sites.

ENGINEERING PIKROMYCIN BIOSYNTHESIS FOR THE PRODUCTION OF NOVEL ANALOGUES

While much of the initial work with type I PKSs involved DEBS, the pikromycin PKS (*Figure 8.2*) from *Streptomyces venezuelae* (Xue *et al.*, 1998) has become a target for generating hybrid polyketides for several reasons. Unlike erythromycin, pikromycin is a ketolide antibiotic and has a 3-keto group, rather than the neutral sugar cladinose. Ketolides are interesting because, unlike erythromycin, they do not induce the MLS_B family of resistance (Bonnefoy *et al.*, 1997) and have been touted as the next generation of macrolide antibiotics (Hunter, 1998). In addition, this PKS system, unlike other macrolide PKSs, produces both 12- and 14-membered macrolactones due to alternative expression of two forms of PikAIV, the final module (Xue and Sherman, 2000) (*Figure 8.2*). The post-PKS tailoring enzymes, DesVII (glycosyl transferase) and PikC (P450 hydroxylase), are able to convert both aglycones to the corresponding bioactive compounds (pikromycin (**1**), narbomycin (**2**), methymycin (**3**), and neomethymycin (**4**)), indicating that both enzymes have relaxed substrate specificity. This unusual flexibility makes the pikromycin system an attractive candidate for use in combinatorial biosynthesis.

The first examples of pikromycin analogues were produced by manipulation of the genes required for the biosynthesis of the amino-sugar, desosamine. The *des* locus contains all of the genes required for the biosynthesis of this sugar from the common primary metabolite, D-glucose-1-phosphate, and attachment to the polyketide backbone (*Figure 8.3*) (Xue *et al.*, 1998). In order to probe the sequence of the desosamine biosynthetic pathway, a series of gene disruption experiments was done by Liu and co-workers. Interestingly, several mutants produced novel pikromycin analogues carrying

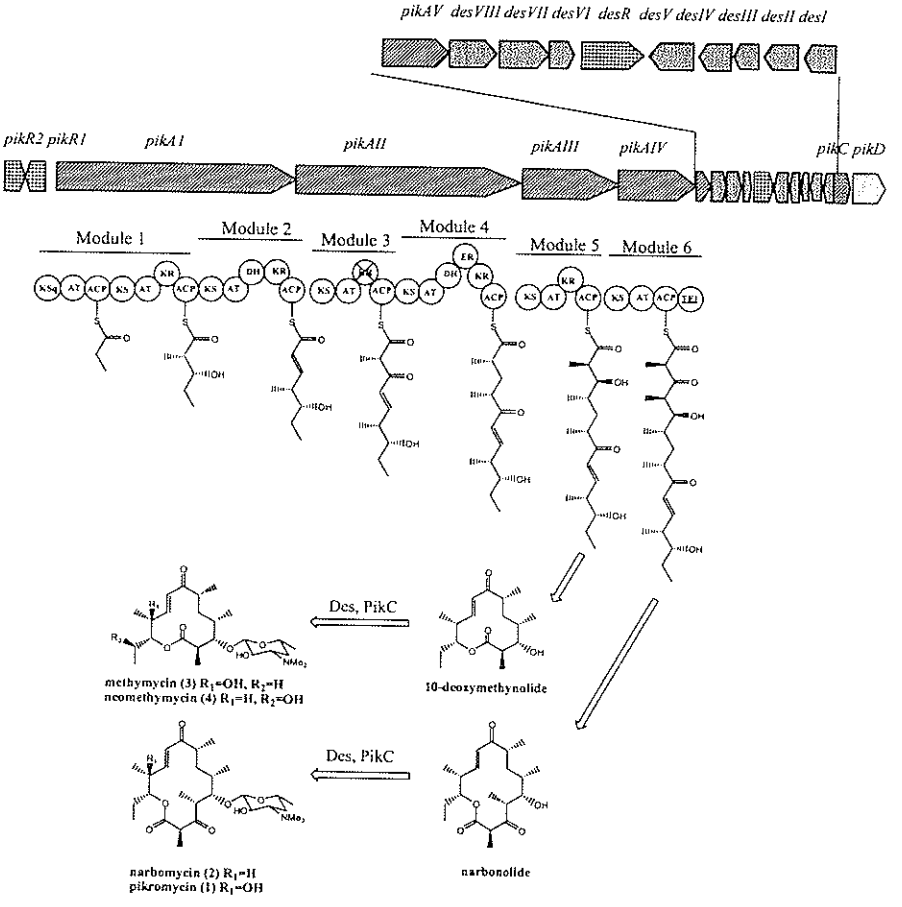

Figure 8.2. Organization of the pikromycin biosynthetic gene cluster, including those encoding the resistance proteins (*pikR*), polyketide synthase (*pikA*), desosamine biosynthetic genes (*des*), P450 hydroxylase (*pikC*), and transcriptional activator (*pikD*).

desosamine biosynthetic intermediates or metabolites thereof. Inactivation of the *desI* gene encoding a PLP-dependent 4-aminotransferase caused the production of an analogue containing D-quivinose in place of desosamine (**5**) (*Figure 8.3*) (Borisova *et al.*, 1999). To further diversify the system in a true 'combinatorial' fashion, *calH*, a gene encoding a 4-aminotransferase from the calicheamicin biosynthetic pathway, was added to the Δ*desI* mutant. In this strain, the accumulated DesI substrate is used by the gene product of *calH*, producing novel pikromycin analogues containing 4-amino sugars (**10** and **11**) (Zhao *et al.*, 1999). Recently, it has been shown that DesI, in concert with DesII, can carry out C-4 deoxygenation through a novel mechanism involving incorporation of a nitrogen functional group at C-4, followed by subsequent rearrangement and elimination of water (Zhao *et al.*, 2001). Interestingly, inactivation

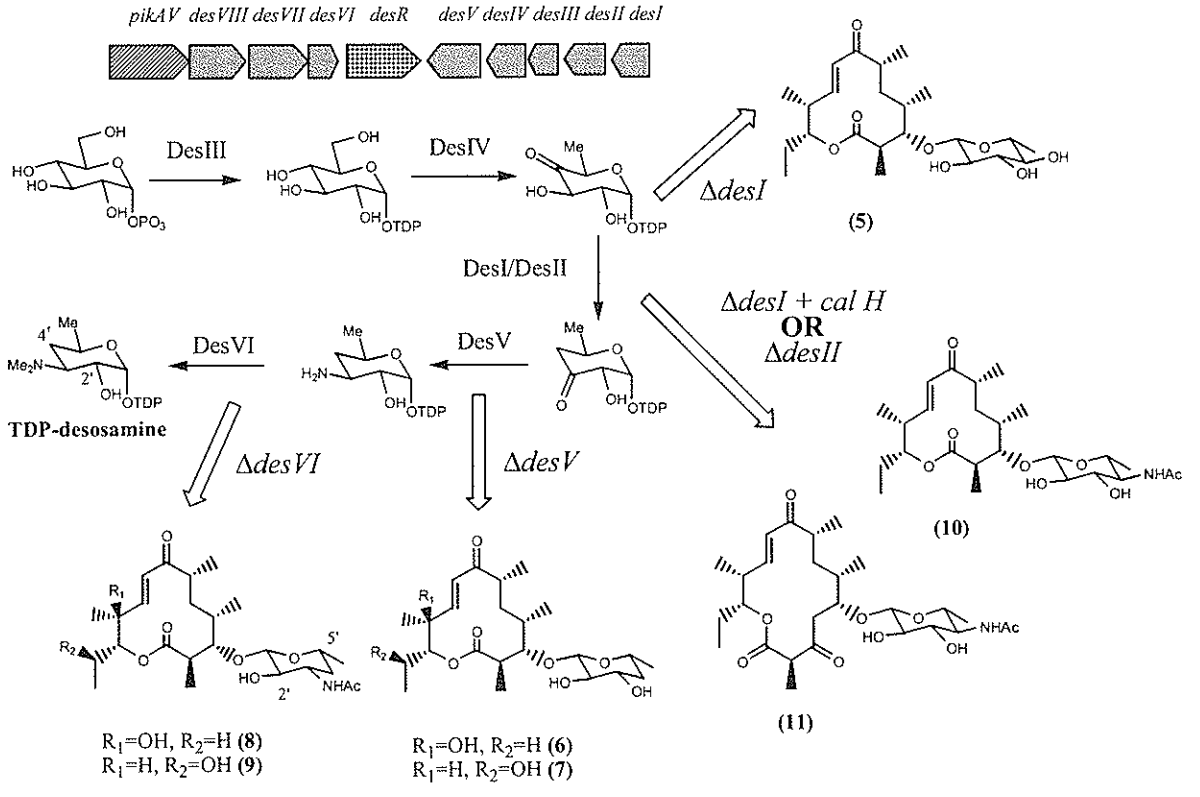

Figure 8.3. Production of novel compounds by engineering the pikromycin gene cluster. Inactivation of the desosamine biosynthetic genes *desI*, *desII*, *desV* and *desVI* yield the corresponding pikromycin analogues containing novel sugar moieties. Addition of *calH* from the calicheamicin biosynthetic gene cluster also produces novel compounds.

of *desII*, which encodes an enzyme believed to be a member of the radical SAM superfamily, produces the same compounds as addition of *calH*, due to the inability to rearrange the product of DesI (Zhao *et al.*, 2001). Inactivation of another aminotransferase, encoded by *desV*, produced analogues containing a 4,6-dideoxyhexose residue (**6** and **7**) (Zhao *et al.*, 1998). Inactivation of *desVI*, encoding an *N*-methyltransferase, produced analogues carrying a 3-*N*-acylated amino sugar (**8** and **9**) (Zhao and Sherman, 1998). It should be noted that, in several of these gene disruption experiments, the expected biosynthetic intermediates do not appear in the novel compounds. For example, in the case of *desVI* inactivation, the sugar moiety is *N*-acylated rather than containing a free amine. Likewise, compounds **6** and **7** do not contain 4,6-dideoxyhexose residues rather than a 3-keto sugar. Presumably, other enzymes, not associated with the desosamine biosynthetic pathway, modify the compounds. The reasons for modifications such as these are unclear, but it may reflect a self-protection mechanism. In fact, many of the pikromycin analogues containing novel sugars are biologically inactive and it could be speculated that the expected products are bioactive but have been detoxified by the host organism. This work, in addition to elucidating the desosamine biosynthetic pathway, indicated that DesVII, the glycosyl transferase, is a very flexible enzyme and is capable of incorporating a variety of unusual sugars on the 12- and 14-membered aglycones produced by the PKS. Another example of the extraordinary flexibility of this system was shown recently by Tang and McDaniel, who used the desosamine biosynthetic pathway of *S. venezuelae* to glycosylate a library of novel polyketides produced by engineered DEBS variants (Tang and McDaniel, 2001).

Novel pikromycin analogues have also been generated by manipulation of the polyketide synthase. This work involved primarily novel subunit replacements using the pikromycin, tylosin and erythromycin PKSs (Yoon *et al.*, 2001). There is some evidence that this approach is superior to individual domain replacements as it introduces whole proteins that are known to work in other systems. In this case, there is less worry that the PKS structure has been altered due to less than optimal fusing of domains or modules (Tang *et al.*, 2000). Using previously described genetic tools (Xue and Sherman, 2001), several complementation experiments aimed at producing novel analogues were conducted (*Figure 8.4*). Using a *pikAI* deletion mutant, DEBS I was introduced with plasmid-based expression of *eryAI* from the constitutive *ermE** promoter. The DEBS I subunit is similar to PikAI, with the exception of module 2, which contains a methylmalonyl CoA specific AT that lacks a DH domain (PikAI contains a malonyl CoA specific AT and a DH domain) (*Figure 8.4*). This strain produced the expected compounds, 8-methyl-9-hydroxy-methymycin/neomethymycin and 10-methyl-11-hydroxy-pikromycin/narbomycin (**12–15**). The same *pikAI* deletion mutant was also successfully complemented with plasmid-based expression of *tylGI*. TylGI is nearly identical in organization to PikAI, with the exception of the opposing stereospecificity of the KR in module 1. In this case, the corresponding pikromycin epimers (**16–19**) are produced (*Figure 8.4*). In addition to altering the chemistry of modules 1 and 2, it was also possible to complement a *pikAIV* deletion mutant to produce novel analogues. Using this mutant, a plasmid expressing *tylGV*, which encodes the final module 7 of tylactone synthase, was introduced. This module differs from PikAIV in that the AT is specific for malonyl CoA, rather than methylmalonyl CoA. This module also contains a KR domain which is absent from

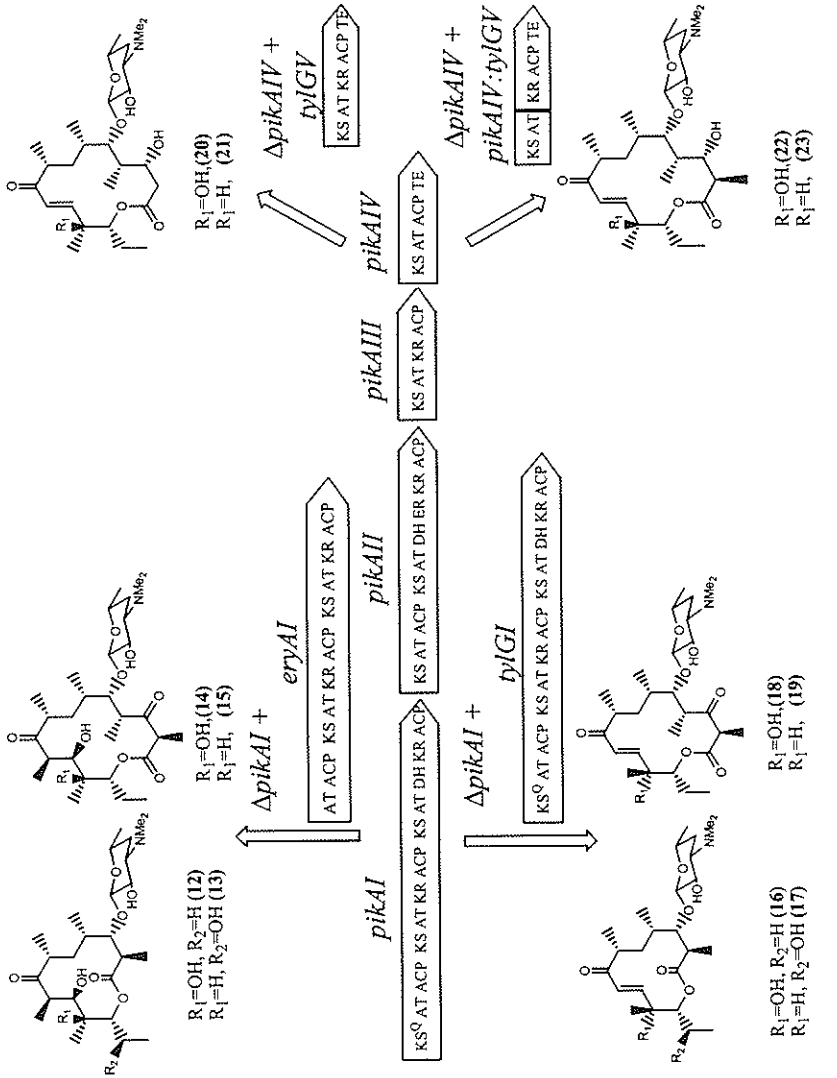

Figure 8.4. Complementation of *pikAI* or *pikAIV* disrupted mutants gives compounds with structural variations in the polyketide backbone.

PikAIV. The expected, novel 14-membered products 2-desmethyl-3-hydroxyl-pikromycin/narbomycin (**20** and **21**) (*Figure 8.4*) were produced. In addition, the natural 12-membered compounds were produced in this strain, suggesting that the presence of TylGV does not disrupt the alternative termination mechanism of the pikromycin PKS. Finally, expressing a hybrid PikAIV containing the natural KS6-AT6 fused to the KR-ACP-TE of TylGV produced the expected compounds 2-desmethyl-narbomycin/pikromycin (**22** and **23**) in addition to methymycin and neomethymycin (Yoon *et al.*, 2001). In this case, expression of a hybrid PKS module showed no real disadvantage when compared to use of the fully intact TylGV.

The results of these experiments exemplify the power of the pikromycin biosynthetic genes of *S. venezuelae* as a tool in combinatorial biosynthesis. Perhaps one of the most important features of this system is that the entire group of novel compounds was glycosylated, displaying the relaxed substrate specificity of DesVII, the single glycosyl transferase in the cluster. The diverse group of compounds produced indicates that this enzyme can accept alterations in either the aglycone or sugar substrate. The yields of these compounds were low, but preliminary analysis of the culture extracts indicated that the compounds containing structural differences in the polyketide backbone exhibited antibacterial activity against *Bacillus subtilis*. In contrast, yields of those containing novel sugar moieties were greater, but the compounds were inactive. Unexpectedly, some of the compounds containing novel sugar moieties were not hydroxylated by PikC, indicating that this enzyme may have substrate specificity for this component of the macrolide structure.

Precursor-directed biosynthesis

While constructing hybrid PKSs holds great promise, it is not the only means for generating new polyketide analogues. Precursor-directed biosynthesis is an alternative approach in which novel precursors are fed to a culture and used by a PKS in place of the endogenous substrates. Typically, the biosynthesis of the normal natural product is disrupted, so the novel precursors are able to reach the PKS without competing with the natural substrates. In the case of DEBS, this has been done very successfully by using site-directed mutagenesis to inactivate KS1, thus abolishing all 6-dEB production (Kao *et al.*, 1996). Using this KS1 null mutant, researchers have been able to feed activated diketide intermediates in the form of *N*-acetylcysteamine (NAC) thioesters and generate a variety of polyketide analogues (*Figure 8.1*) (Jacobsen *et al.*, 1997; Hunziker *et al.*, 1999; Kinoshita *et al.*, 2001). This method makes it possible to generate a variety of erythromycin analogues, which are produced in reasonable yields and have more substantial structural changes than can be obtained using a hybrid PKS. The major caveat with this approach, however, is that diketide NAC derivatives are not commercially available and must be obtained through multi-step syntheses.

An example of precursor-directed biosynthesis which has resulted in the generation of a new commercial polyketide product has been described with *Streptomyces avermitilis*, which produces the family of anthelminic polyketides known as the avermectins. The avermectin PKS contains a loading module responsible for loading either isobutyryl CoA or 2-methylbutyryl CoA, which give rise to the 'b' and 'a' avermectins, respectively (*Figure 8.5*). These branched-chain starter units are catabolites

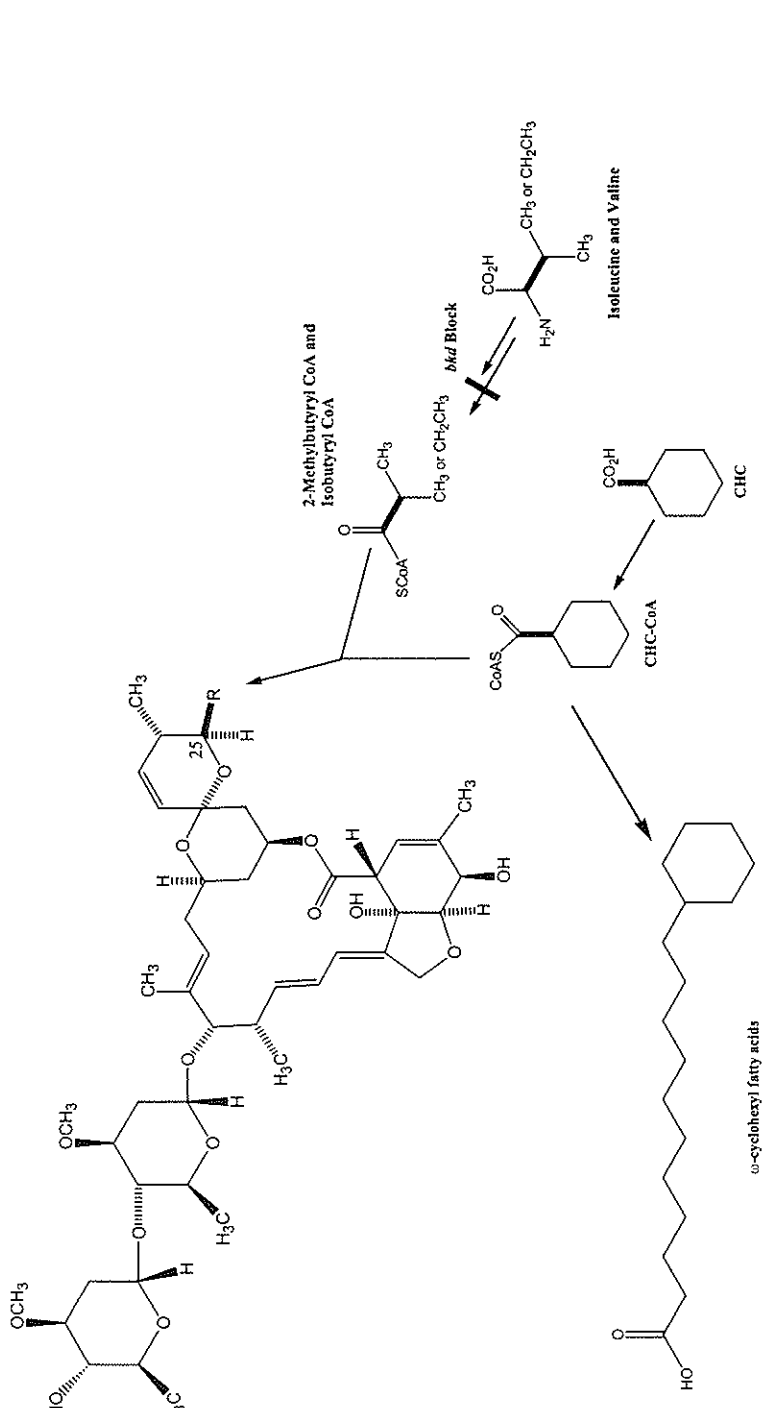

Figure 8.5. The role of precursors in the biosynthesis of fatty acids, avermectin, and doramectin in *S. avermitilis*. Avermectin B1a and B1b are generated using the starter units 2-methylbutyryl CoA and isobutyryl CoA, respectively. In the *bkd* mutant of *S. avermitilis*, the formation of these starter units from branched-chain amino acid degradation is blocked. Doramectin B1 is generated using CHC-CoA as a starter unit. This starter unit can either be obtained by addition of CHC to fermentations of *S. avermitilis* or by introduction of the CHC-CoA biosynthetic gene cassette (pAC12).

amino acids, valine and isoleucine. The fact that this module possesses a relaxed substrate specificity prompted researchers to investigate the possibility of incorporating exogenous carboxylic acids in place of the normal starter units. More than 60 avermectin analogues were generated simply by feeding precursors to a mutant blocked in the production of the natural starter units by inactivation of the branched-chain α-ketoacid dehydrogenase complex (*Δbkd*) (Dutton *et al.* 1991; Denoya *et al.*, 1995). In this case, the unnatural primer unit is fed to the culture as a free acid, taken up by the cell, where it is presumably activated by a non-selective acyl CoA ligase and loaded onto the PKS, giving rise to the corresponding C25 analogue. In addition to producing novel avermectins, the organism also produced fatty acids arising from the unnatural starter unit, such as cyclohexane carboxylic acid (CHC) (*Figure 8.5*) (Cropp *et al.*, 2000a). The novel avermectin analogue derived from a CHC primer unit was also found to be the most efficacious, and went on to become the industrially important doramectin (McArthur, 1998), sold under the trademark Dectomax.

USE OF A CYCLOHEXYLCARBONYL CoA BIOSYNTHETIC GENE CLUSTER TO PRODUCE DORAMECTIN

The production of doramectin is an example of producing an industrially important pharmaceutical by precursor-directed biosynthesis. The process is not perfect, however, and still requires the addition of exogenous CHC to the fermentation. Furthermore, the novel precursors must cross the cell membrane and be activated as CoA thioesters in order to serve as starter units for the PKS. Obviously, an attractive alternative to precursor feeding would be to produce a strain which is capable of generating activated CHC-CoA *in situ*. An example of this is in *Streptomyces collinus*, where activated CHC-CoA is generated in the biosynthesis of ansatrienin A. This interesting biosynthetic pathway generates CHC-CoA from endogenous shikimic acid in nine enzymatic steps (*Figure 8.6*) (Moore *et al.*, 1993). Purification and characterization of 1-cyclohexenylcarbonyl CoA reductase (ChcA) showed that not only was this enzyme involved in the pathway, but also that it was able to catalyze several of the reductive steps as well (Wang *et al.*, 1996). Sequencing of the *chcA* gene demonstrated that it was clustered in a putative CHC-CoA biosynthetic gene cluster along with four other ORFS (*ansJ–M*), adjacent to the ansatrienin PKS (*Figure 8.6*) (Chen *et al.*, 1999). Sequence analysis suggested that these ORFS (*ansJ–M*) may encode the other enzymes involved in the pathway. This prompted us to construct a gene cassette (pAC12) containing all of the genes putatively required for CHC biosynthesis downstream of the constitutive *ermE** promoter on an *E. coli*:*Streptomyces* shuttle vector (Cropp *et al.*, 2000b). This construct would allow us to move the pathway into a heterologous host to provide CHC-CoA for processes other than ansatrienin A biosynthesis. The biosynthesis of doramectin presented an excellent system in which to test this hypothesis.

Initially, pAC12 was introduced into *Streptomyces lividans* 1326, as it is much more amenable to genetic manipulation than *S. avermitilis*. Plasmid-based expression of the cluster was verified by ChcA assays using 1-cyclohexenylcarbonyl CoA as a substrate. In the *S. lividans* strain carrying the pAC12 plasmid, ChcA activity was found to be 16-fold greater than *S. collinus*. The high level of ChcA expression (which is the fourth ORF downstream of the *ermE** promoter) was consistent with the other

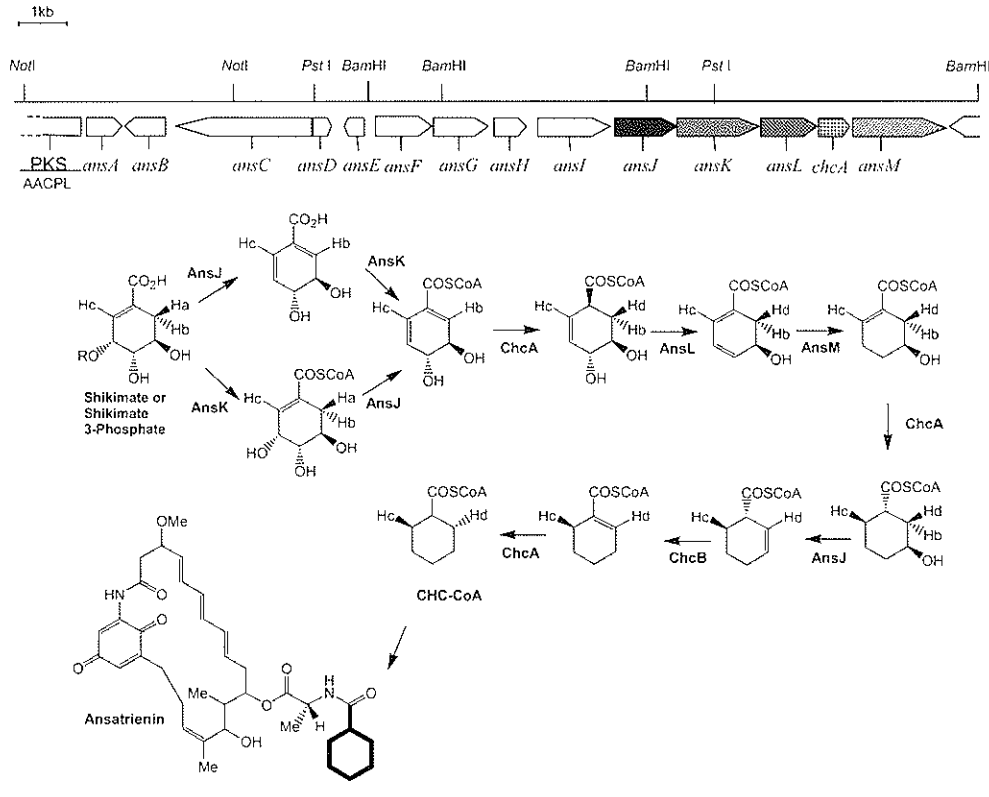

Figure 8.6. The CHC-CoA biosynthetic genes of the ansatrienin biosynthetic gene cluster. Proposed functions of *ansJ*, *ansK*, *ansL*, *chcA* and *ansM* (shaded) gene products in the CHC-CoA biosynthetic pathway are shown.

four ORFs also being expressed (Cropp *et al.*, 2000b). In addition to high level expression of ChcA, the *S. lividans* 1326/pAC12 strain also produced ζ-cyclohexylfatty acids, derived from a CHC-CoA starter unit (*Figure 8.5*). When pAC12 was introduced to the *bkd* mutant of *S. avermitilis*, the resulting strain produced both ζ-cyclohexylfatty acids and doramectin without exogenous CHC. The application of this CHC-CoA biosynthetic gene cluster to the production of doramectin represented one of the first examples of combinatorial biosynthesis combining a precursor pathway from one organism with a PKS system from another. This system could be used to supply CHC-CoA, or pathway intermediates, to serve as novel building blocks for other PKSs.

Conclusion

In this review, we have presented a few examples of approaches for obtaining novel polyketides through combinatorial biosynthesis. As described, approaches can involve engineering PKSs, sugar biosynthetic processes, and precursor supply. Methods such as these are being used to generate appropriate analogues for either improved activity/decreased toxicity or introduce chemical 'handles' on which to perform additional synthetic chemistry. Alternatively, this approach can be used to provide combinatorial libraries, which can then be screened for novel or improved bioactivities. In the example given, only one 'layer' of combinations has been generated. There are a large number of compounds that could be accessed by an additional layer of combinatorial biosynthesis involving structural modifications in more than one region of the molecule. Using the pikromycin system for example, mixing all of the novel sugar residues with the hybrid polyketide aglycones, would increase this size of this library substantially. Likewise, precursor-directed biosynthesis, such as that used for the discovery of doramectin, could also be added to give another layer of structural diversity. Combinatorial biosynthesis is a relatively new and useful tool, the full potential of which, in the quest for discovering novel natural products, has yet to be fully exploited.

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