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Combinatorial biosynthesis on Polyketide Synthase type I

Anita Minnen, review supervised by Stefan Kol
Microbial physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, The Netherlands

Abstract

Using an antibiotic in the clinic will inevitably lead to resistance of bacteria. Because untreatable bacterial infections will lead to worldwide health problems there is an ongoing need for new antibiotics. Several techniques are available to aid in the development of novel antibacterial compounds. Combinatorial biosynthesis of modular type I Polyketide Synthases (PKSs) is one of these techniques. The main aim of this review is to investigate applied and novel combinatorial approaches. We do this by describing the successes and challenges in combinatorial approaches of the extensively studied erythromycin PKS. We will compare our findings to the less studied tylosin PKS. Furthermore, we will seek novel combinatorial approaches that could be applied to tylosin PKS in order to find novel antibiotic derivatives of tylosin.

Abbreviations

PKS	polyketide synthase
DEBS	6-deoxyerythronolide B Synthase
6dEB	6-deoxyerythronolide B
DEBS 1	subunit 1 of DEBS
DEBS 2	subunit 2 of DEBS
DEBS 3	subunit 3 of DEBS
AT	acyl transferase
ACP	acyl carrier protein
KS	keto synthase
KR	keto reductase
DH	dehydrase
ER	enoyl reductase
TE	thioesterase

Introduction

Many will have found them self going to the doctor with a problem that later turned out to be a so called ‘bacterial infection’. Most people get an antibiotic prescribed by their doctor, return home, take their medicine and will be healthy a few weeks later.

What many people do not know is that a part of the bacterial population might become resistant to the used antibiotic and that these bacteria survive the treatment. This is due to the development of an intrinsic or acquired resistance mechanism of the bacteria. Subsequently, this little group of resistant bacteria will grow into a large population causing their host to become ill once again. Other antibiotics will be applied, causing these bacteria to become resistant again, which in turn leads to multi-drug resistant bacteria that are no longer susceptible to any available antibiotic. When these bacteria are spread, large amounts of people can become infected and get ill. Therefore, there is an ongoing need for new antibiotics. Fortunately, to date all harmful bacteria are treatable with at least one antibiotic.

A famous example of a bacterial threat to human kind in this respect is the bacterium *Staphylococcus aureus.* Its infections were easily treatable in the 1940s with penicillins. Resistance occurred within a year and methicillin was used to treat infections. By 1986 methicillin-resistant *S. aureus* (MRSA) was so abundant that MRSA infections were treated with vancomycin, leading to the vancomycin-resistant *S. aureus* (VRSA). In the late 90s two compounds (Synercid and Zyvox) were admitted as new antibiotics against this dangerous multi-drug resistant bacterium (Walsh 2003a). By 2008 more compounds were approved to treat *S. aureus* infections, but they have to be applied cautiously (Sievert *et al.* 2008).

To defeat multi-drug resistant bacteria that are no longer susceptible to any antibiotic compound there is an ongoing need for new antibacterial compounds. Although novel antibiotics will inevitably lead to resistance, these compounds might be effective for a period of time varying from one year to over a decade in clinical use (Walsh 2003b).

For a compound to be a good antibiotic it has to be toxic to microbes but not for the host. In general this means that antibiotics are targeted at specific mechanisms that only microbes carry (Walsh 2003a). Antibiotics come in different shapes and sizes of which the majority will be mentioned. Two categories are distinguishable; synthetic agents and antibiotics occurring naturally. The synthetic agents harbor the so-called sulfa drugs that act on the folate metabolism. They act by inhibiting the formation of thymine, a compound which is essential to replicate DNA. Other examples of synthetic agents are quinolone compounds which are characterized by a quinoline ring and inhibit DNA gyrases (Madigan & Martinko 2006).

Naturally occurring antibiotics harbor many more compounds. Carbohydrate-containing compounds include pure sugars, aminoglycosides, orthosomycins, N-glycosides, C-glycosides and glycolipids. Another group of naturally occurring antibiotics are quinones. This group resembles a cyclic organic compound, containg two carbonyl groups in a six-membered unsaturated ring. Tetracyclines are part of this group and their mechanism of action involves inhibition of protein biosynthesis by binding to the 30S ribosomal subunit. Amino acid and peptide analogs contain one of the most famous antibiotic,

penicillin. This compound is a so-called ß-lactam and inhibits cell wall synthesis (Madigan & Martinko 2006).
Heterocyclic compounds containing either nitrogen or oxygen are two other examples. Alicyclic derivatives, aromatic compounds, aliphatic compounds which contain phosphorus and oxazolidinones are other examples. Macrocyclic compounds resemble, amongst others, macrolide antibiotics which have large lactone rings connected to sugar molecules (Madigan & Martinko 2006). Polyketides belong to this group of which erythromycin and tylosin are examples. These examples will be the main focus of this review.

Polyketides
Polyketides are secondary metabolites produced by bacteria, fungi and plants (Shen 2003) They are of interest due to their antibiotic, immunosuppressive, anti-parasitic, anti-fungal, cholesterol lowering and anti-cancer activities (Weissman & Leadlay 2005, Staunton & Weissman 2001). The biosynthesis of polyketides strongly resembles fatty acid biosynthesis. Polyketides are produced by polyketide synthases (PKSs) of which three types are distinguishable; PKSs type I are large enzyme complexes that can be organized in two ways; Iteratively whereby different domains are reused in a cyclic fashion. Secondly, in a modular fashion in which protein subunits are divided into modules (Weissman 2004). PKSs type II produce aromatic polyketides and consist of dissociated enzymes. A type II PKS gene cluster contains genes which encode a PKS with tailoring enzymes (Zhang & Tang 2009). PKSs type III consist of only one protein which is a homodimeric condensing enzyme that acts iteratively. (Watanabe *et al.* 2006); PKSs type I will be the main focus of this review.
A type I PKS consist of several subunits which contain several modules. These modules are made up of a few domains. The entire gene cluster sequence of a modular type I PKS, erythromycin, was elucidated in 1990 (Weissman 2004) and revealed the clustered organization of the erythromycin PKS and modifying post-PKS enzymes (Weismann & Leadlay 2005). Moreover, in bacteria and fungi, with a few exceptions, all PKS genes and post-PKS genes are clustered including the regulatory genes and genes for self-resistance (Weismann & Leadlay 2005). This and the fact that PKSs are organized in a modular way makes them excellent candidates for combinatorial biosynthesis. Hereby different components of type I PKSs, domains, modules or even subunits, can be exchanged. This rearrangement hopefully leads to the production of novel (bioactive) compounds (Weissman 2004).
In this review, the characteristics of a modular type I PKS will be discussed with erythromycin as an example. The main aim is to determine which combinatorial biosynthesis approaches have been performed and which other combinatorial biosynthesis approaches are possible on type I PKSs to create novel compounds. We will concentrate on one example, the antibiotic tylosine which is synthesized by a modular type I PKS. Erythromycin PKS produces the antibiotic erythromycin and has been studied extensively with regard to combinatorial biosynthesis. The tylosine PKS has been less studied and will be compared to erythromycin PKS with regard to the combinatorial approaches that have been applied to tylosin PKS and erythromycin PKS. In short, this review asks the question whether the combinatorial techniques that have been applied to erythromycin PKS can possibly be applied to tylosin PKS.

Erythromycin
Erythromycin is a 14-membered macrolide antibacterial compound produced by *Saccharopolyspora erythraea*. The entire erythromycin PKS (or 6-deoxyerythronolide B synthase, (DEBS)) is divided over three enormous proteins called DEBS 1, 2 and 3 (fig. 1). The different domains of this PKS are covalently linked and organized into six modules (fig. 1). Every single module contains the enzymes essential for polyketide elongation, with one building block, and the necessary processing.

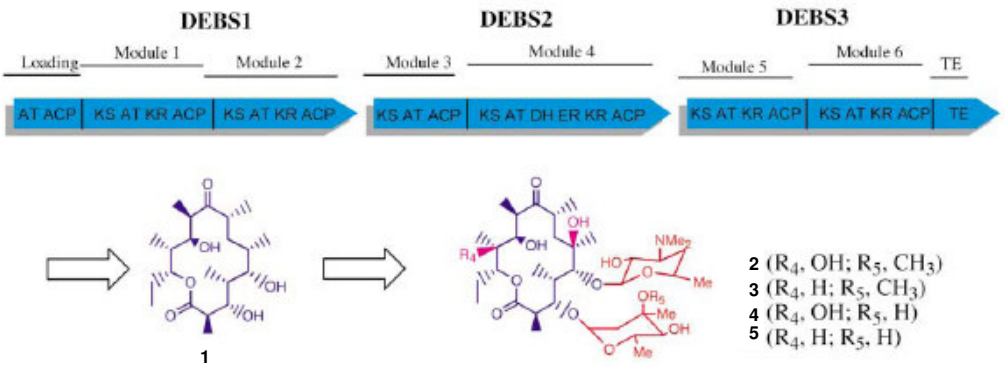

Figure 1. Organization and products of DEBS.
DEBS consist of three subunits (DEBS 1, DEBS 2 and DEBS 3) which contains different modules which in their turn consist of different domains. The end module is indicated by a TE domain. The obtained product is 6-deoxyeythronolide B (6dEB) (1) which is converted to erythromycin A, B, C and D (2, 3, 4 and 5) by post-PKS processing.
Adapted from Yoon *et al.* 2002

Subsequently, the polyketide is transferred to the next module. Besides these six elongation modules there are two additional modules; a loading module and an end module. The loading module is positioned in front of the elongating modules and initiates the synthesis of a polyketide. In this case an acyl transferase (AT) domain selects the starter unit, propionate, and binds this covalently to an acyl carrier protein (ACP) in the loading module. The ACP delivers the molecule to the appropriate enzymes in the next module. Transacylation takes place to the keto synthase (KS) domain of elongation module 1 where chain elongation begins (Donadio & Sosio 2003). Every elongation module consists of an AT, KS, ACP domain and one or more reductive domains (keto reductase (KR), dehydrase (DH) and enoyl reductase (ER)). The building blocks are selected by six ATs that are methylmalonate specific, the required new carbon-carbon bonds are formed by the KS that carries out a so called Claisen condensation. A KR is present in each elongation module to carry out reductive processing, in some modules a DH and an ER are present to carry out additional reductive processing. Every elongation module contains an ACP that acts like a flexible arm which ensures that the incomplete polyketide finds the correct catalytic site of an enzym. The end module consists of a thioesterase (TE) that terminates chain construction by cyclization.
The PKS forms a polyketide intermediate. To obtain the bioactive compound, further post-PKS processing is necessary. In this case the aglycone 6-deoxyerythronolide B (6dEB) is released from the TE. Subsequently two 6-deoxysugars are attached; D-desoamine at C5 and L-cladinose at C3. The product is hydrolyzed twice (at C6 and C12) and methylated on a sugar residue. This leads to the completion of the synthesis of erythromycin A (fig.1) (Weissman & leadlay 2005, Weissman 2004, Staunton 1998, Katz 1997).

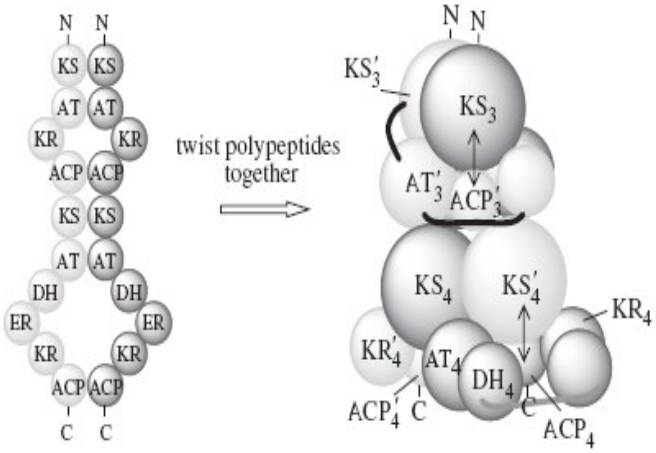

Figure 2. ´Cambridge´ double-helix model for the structure of DEBS
Each subunit consists of two identical polypeptides that twist around each other, the KS, AT and TE domains are dimers, the reductive domains form loops. The KS of one subunit cooperates with the ACP on the subunit, the arrows indicate this.
Adapted from Weissman 2004

The three-dimensional structure of a type I PKS has not been determined so far, but the structures of a few domains are clarified. Models have been proposed for DEBS whereof the so called 'Cambridge' model is adapted as a working model. This model states that the PKS consists of two copies of every subunit which are twisted around each other forming a double helix (fig. 2). The KS, AT and TE domains are dimers whereas the reductive domains do not interact with each other (Weissman 2004).

Combinatorial biosynthesis on erythromycin PKS
It would go beyond the scope of this review to give an entire overview of the combinatorial experiments that have been applied to erythromycin. Therefore, a few approaches with examples should give an idea of the possibilities.

Chain length alteration
The number of modules in a PKS type I determines the chain length of a polyketide. Changing the number of modules can therefore change the chain length. In various experiments with erythromycin the chain length has been shortened. In these experiments the TE domain is placed after a preceding module. This has been done by placing the TE domain after module 2, 3 and 5 whereby a tri-ketide, tetraketide and a 12-membered macrolactone, respectively were formed (Donadio & Sosio 2003, Staunton 1998).
Extending the chain length seems to be more difficult but at least one experiment shows that it is feasible. Rowe and coworkers have shown the insertion of module 2 of the rapamycin PKS into DEBS1 TE. In DEBS 1 TE, the TE domain of DEBS is placed after module 2 of DEBS. Module 2 of rapamycin PKS was inserted between module 1 and 2 of DEBS leaving the linker regions of DEBS intact. These mutants produced the expected tetraketide. The endologous triketide was produced as well and the ratio of the tetraketide:triketide was 1:20. This suggest that the inserted module had not been used consequent in chain elongation, a phenomenon Rowe refers to as 'skipping' (Rowe *et al.* 2001).
Chain elongation can happen spontaneous by iterative use of one module. This has been reported for *S. erythraea* which produces a 16-membered macrolactone (instead of the 14-membered 6dEB) by iterative use of module 4. This compound is produced in very small amounts (1% of 6dEB) (Donadio & Sosio 2003). In principle this 'technique' is not part of combinatorial biosynthesis since no combinatorial techniques have been applied, but when this iterative use of one module is consistent, one can think of applications in combinatorial biosynthesis.

Swapping domains, modules and subunits
Another combinatorial approach is to swap one domain or module from one PKS into another. Hereby a domain or module of one PKS is replaced by a domain or module of another PKS. In most cases this will change specificity. An approach that has been used extensively is to change the

AT domain. This has been applied to all elongating modules whereby they changed from methylmalonate specific into malonate specific modules. The obtained products were indeed desmethyl analogs of 6dEB.
One of the first examples of this approach was the transplant of an AT domain from rapamycin PKS into DEBS 1 TE. Hereby the existing methyl-malonate specific AT of module 1 was exchanged for a malonate-specific AT. The product was isolated and indeed lacked the methyl group at the expected site (Olynk *et al.* 1996, Donadio & Sosio 2003, Ruan *et al.* 1997, Liu *et al.* 1997, McDaniel *et al.* 1997).
Only one change from methylmalonate into ethylmalonate has been reported so far. Into module 4 of DEBS the AT domain was replaced with the ethylmalonate specific AT domain of module 5 from niddamycin PKS. This resulted in the production of the predicted 6-desmethyl-6-ethyl 6dEB derivative after engineering the *S. erythraea* strain to produce enough intracellular amounts of ethylmalonate (Donadio & Sosio 2003).
In addition to this it has been shown that whole subunits can be substituted as well. This was done by replacing modules 5, 6 and the end module (subunit 3) of picromycin PKS with their counterparts of DEBS (DEBS 3). The obtained product was 3-hydroxy-narbonolide (Tang *et al.* 2000).

Changing reductive states
A further strategy is to change the reductive state of compounds. This was done by either inactivating one of the reductive domains (KR, DH or ER) or adding one or more domains. Inactivation of reductive domains in DEBS has been carried out by deleting the KR domain of module 5 and 6 (Donadio *et al.* 1991, McDaniel *et al.* 1999). These mutants produced the expected compound which contained a keto group at positions 5 and 3.
Another experiment showed the inactivation of module 4 by site-directed mutagenesis. This led to the production of an analog of 6dEB wherein position 6-7 contained a double bond (Donadio *et al.* 1993). Questionable is whether this approach is part of combinatorial biosynthesis, since no combinatorial techniques are applied. Instead domains are deleted or inactivated. This approach is strongly related to the techniques of combinatorial biosynthesis and one can think of other (combinatorial) ways to achieve this, e.g. replacing a reductive domain by a nonreductive domain, leaving the linker domains intact. Therefore, we will consider this approach as a part of combinatorial biosynthesis.
By using techniques to inactivate a domain, the level of oxidation was increased. Addition of one or more reductive domains will lead to a change in the reductive state of a compound. In DEBS this has been performed in several ways. A DH-KR segment of the rapamycin PKS was placed in modules 2, 5 and 6 of DEBS, the KR domain of DEBS was hereby replaced. This led to the production of 6dEB derivatives which were unsaturated at position 10-11, 4-5 and 2-3, respectively. Another example of this approach is replacing the DH, ER, KR segment from module 1 of the rapamycin PKS into modules 2 and 5 of DEBS. The produced compounds contained methylene groups at position 11 and 5, respectively (Donadio & Sosio 2003).

Changing starter unit
Another interesting approach is to change the starter unit. An example of this is the replacement of the loading module (both the AT and ACP domain) of DEBS by the loading module of avermectin PKS. This led to the production of novel compounds in which the starter units of avermectin PKS (2-methylpropionate and 2-methylbutyrate) were used instead of the starter unit of DEBS (Marsden *et al.* 1998, Donadio & Sosio 2003).
From all these approaches novel compounds were produced, some with bioactive properties. Unfortunately, none of them made it to clinical use. Furthermore, there are many challenges posed by attempting to successfully apply combinatorial biosynthesis on polyketide synthases. These challenges will be considered here.

Challenges
Host-cells
First of all host-cells need to be resistant to the novel compound they produce, working with polyketide antibiotics this is not always the case. Host cells usually carry a resistance gene for the polyketide antibiotic they produce, but with an altered polyketide the resistance gene might not have the correct specificity. In addition to this the host cell needs to be well equipped with the necessary building blocks and enzymes. These are necessary to activate the PKS proteins by attaching the flexible arm to an ACP.

PKS domains
The PKS domains give rise to many challenges. To be useful in combinatorial biosynthesis they must have certain features. An AT should preferably use one exclusive starter unit and transfer a building block to any ACP. A KS must be able to partner with any ACP and accept any incoming chain and building block. A KR should exclusively and irrespectively of the substrate structure carry out an ‘A-type’ or ‘B-type’ ketoreduction. DH and ER should function on any substrate. An ACP should be recognizable for all the other domains in the module. Finally the TE should perform regiospecific macrolactonization (Weissman 2004).

Substrate specificity
Substrate specificity is an important feature since combinatorial biosynthesis will cause unnatural polyketide chains to encounter endologous PKS domains. These have to be able to process them in their natural way. Therefore, in combinatorial biosynthesis, domains with low substrate specificity are preferred. This accounts especially for the

KS, KR, DH, ER and TE domains because they process a substrate that has been altered by previous domains and modules. On the contrary, an AT domain has to select the correct building block and should therefore have a high substrate specificity to produce only one specific polyketide. It is important to know these substrate specificities and find domains that are ideal to use in combinatorial biosynthesis. If these domains can not be found, one might think of altering the specificity by engineering techniques, thereby creating domains that have low substrate specificity. Overall, knowledge about the specificity of the different domains is important and will help in using combinatorial biosynthesis and other approaches to produce novel compounds (Weissman 2004).

Linker regions

Besides this challenge another complication arises; linking the domains, modules and subunits. In an endogenous PKS, domains are linked by 20 to 250 amino acids referred to as linkers. Two options of interacting with these linkers are possible, first joining the domains within the linker regions whereby the linker regions of the 2 adjacent domains are combined. Secondly, joining the domains at the beginning and/or end leaving the linkers intact (Weissman 2004). When these linkers do not have the appropriate properties the protein might fold differently resulting in a non-functional protein. For example, the three dimensional structure between the domains might alter resulting in domains that can not interact with each other. For instance; an ACP must be able to transfer the polyketide chain to the next domain, when inappropriately organized a domain might be skipped.

Considering this, it might be easier to take whole modules as combinatorial units since the domain linkers will stay intact. Unfortunately these modules are linked by linker regions as well and give rise to the same problem. Investigating the possibility to develop universal 'interdomain' or 'intermodular' linkers that can link any part of PKS would therefore be very useful (Weissman 2004).

Docking domains

The same strategy could be applied to the linker regions between different proteins of a PKS (e.g. for DEBS this were DEBS 1, 2 and 3 see fig. 1) which are so-called 'docking domains'. Besides linking to the correct partner protein, they also have the fold in a correct way to form the PKS that produces the desired product. Therefore, developing docking domains which can be applied to different proteins would be very convenient. In recent years progress has been made in understanding the docking mechanism (Buchholtz 2009, Weissman 2006). For example, the docking structure of DEBS2 and DEBS3 has been clarified. Thought is that only 12 residues interact in the actual docking, if this is the case only these residues have to be changed in order to make docking between different docking domains possible (Weissman 2006). In this case, the docking domains do not have to be transferred completely and probably the three dimensional structure will remain intact. Unfortunately, nearly every docking domain and its corresponding mechanism is different. More insight in the mechanism of docking will improve combinatorial approaches.

Three dimensional structure

Another challenge is the three dimensional structure. Stated above is the working model for DEBS, the 'Cambridge' model. Although experiments show that this model seems to be correct, more structural information is needed to fully understand how the linker regions and docking domains function (Weissman 2004). Revealing

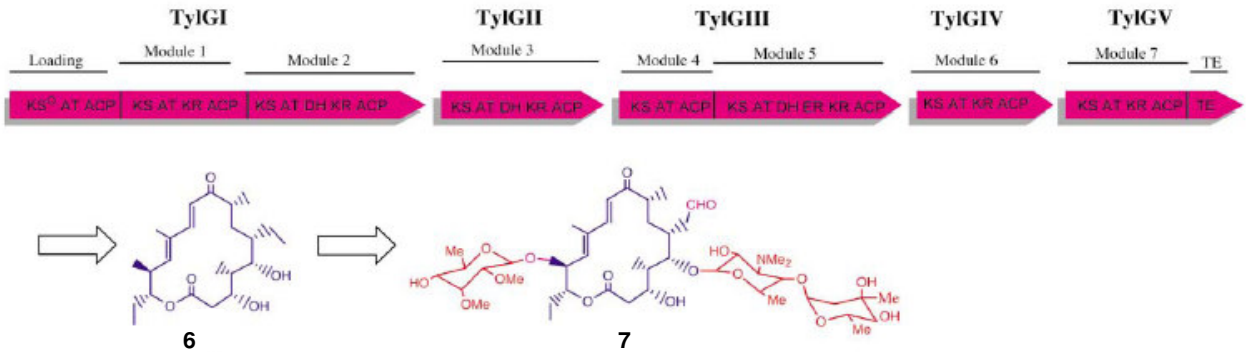

Figure 3. Organization and products of Tyl PKS

Tyl PKS consist of five subunits (TylGI, TylGII, TylGIII, TylGIV, TylGV) which contain different modules which in their turn consist of different domains. The end module is indicated by a TE domain. The obtained product is tylactone (6) which is converted to tylosin (7) by post-PKS processing.

Adapted from Yoon *et al.* 2002

this structure will also lead to a better understanding of the substrate specificity of separate domains and how the polyketide chain is transported through the PKS.

Post-PKS processing

A final challenge is the post-PKS processing. When a polyketide intermediate has been formed by a PKS, the post-PKS processing determines the final compound. When enzymes active in this process are unable to carry out their activities, (because of an altered polyketide) an unexpected compound might be formed. Although this could lead to a novel compound, it is not the desired way. Just as we need to understand the structure of PKS it would be equally useful to understand the post-PKS processing with the enzymes involved and their mode of action (Weissman 2004).

With all these challenges in mind it is not hard to imagine that successful combinatorial biosynthesis approaches do not abound and when novel compounds are produced their yields are usually not very high. The search for novel compounds by combinatorial biosynthesis is ongoing, but most of the recent research is more focused on understanding the mechanisms of the PKS (Buchholtz *et al.* 2009).

Tylosin

Erythromycin has been studied extensively and there is no other PKS on which so many combinatorial biosynthesis approaches have been applied. Therefore, we will compare the less studied macrolide antibiotic tylosin to erythromycin in order to explore future perspectives suggested by this antibiotic.

Tylosin is a polyketide produced by *Streptomyces fradiae*. The polyketide intermediate produced by the tylosine PKS (tyl PKS) is a 16-membered macrolide called tylactone (6) (fig. 3). The tyl PKS consist of 5 subunits, 7 elongation modules, one loading module and one end module (fig. 3) (Reeves 2004). The starter unit of tyl PKS is propionate, the elongation unit is malonate (Kuhstoss 1996). After the formation of tylactone, post-PKS processing involves addition of mycaminose to C-5 and 6-deoxy-D-allose to C-23, addition of mycarose to mycaminose, hydroxylation of C-20 and C-23, dehydrogenation of C-20, addition of a methyl group to demethylmacrocin and macrocin by which tylosin (7) is obtained (Baltz *et al.* 1982).

Combinatorial biosynthesis on tylosin PKS

The very few combinatorial approaches, in comparison to erythromycin, that have been performed on tylosin will be discussed here. Reeves and co-workers carried out an approach whereby subunits of chalcomycin PKS (which synthesizes a 16-membered macrolide) and tyl PKS were combined. Hereby subunits 1 and 2 of chalcomycin PKS and subunits 3-5 of tyl PKS were joined. The C terminal docking domain of tyl PKS on subunit 2 was swapped with the chalcomycin equivalent to optimise the interaction between the subunits. The obtained product was the expected chalcolactone-tylactone hybrid; 12,16-didesmethyl-demycinosyltylosin. This indicated that the post-PKS processing took place as with the native tylosin (Reeves *et al.* 2004).

Domains, modules, subunits and genes from tyl PKS have been used in other combinatorial approaches. Yoon *et al.* gives a good example of this; using pikromycin PKS as a starting point they performed several combinatorial approaches, mostly to investigate the *Streptomyces venezuelae* pikromycin pathway (fig. 4) (Yoon *et al.* 2002). Entire subunits and domains were swapped. One approach was the substitution of pikromycin PKS subunit 4 with subunit 5 (module 7 + TE domain) of tyl PKS leading to the formation of 2-desmethyl-3-dihydro-narbonolide (14). Post-PKS processing involved Des enzymes (responsible

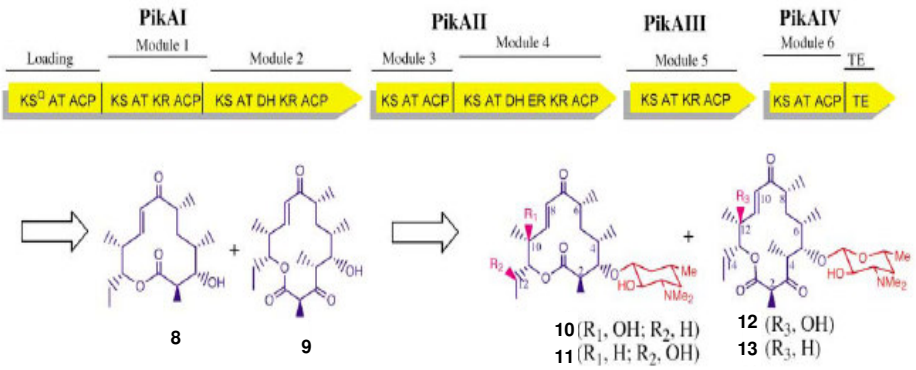

Figure 4. Organization and products of Pikromycin PKS

Organization and products of Pikromycin PKS

Pikromycin PKS producing 10-deoxymethynolide (8) and narbonolide (9) after post-PKS processing methymycin (10), neomathymycin (11), pikromycin (12) and narbomycin (13) are present.

Adapted from Yoon *et al.* 2002

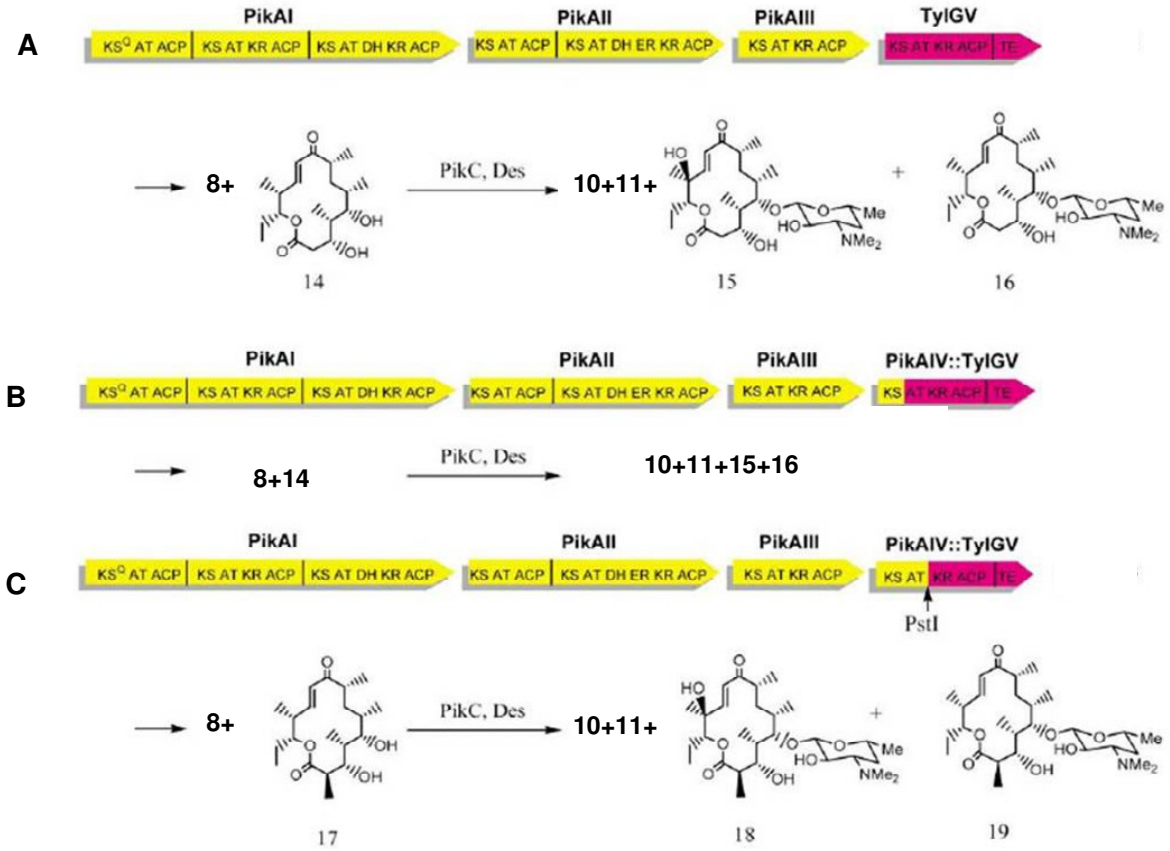

Figure 5. Combinatorial approaches on pikromycin PKS with segments of tylosin PKS as substituent.
Domains of pikromycin PKS are marked in yellow, domains of tyl PKS are marked in red.
Adapted from Yoon *et al.* 2002

for desosamine biosyntheses and transfer) and Pik C (cytochrome P450 monooxygenase, responsible for hydroxylation) and resulted in two compounds; 2-desmethyl-3-dihydro-pikromycin (15) and 2-desmethyl-3-dihydro-narbomycin (16) (fig. 5A) (Yoon *et al.* 2002). Another experiment was the substitution of pikromycin PKS AT and ACP domain of module 6 and TE with the AT KR and ACP domain of module 7 and TE of tyl PKS. This led to the production of the same compounds as in the previous approach (fig. 5B) (Yoon *et al.* 2002).

Another combination was made with the substitution of pikromycin PKS ACP domain of module 6 and TE domain with the KR and ACP domain of module 7 and TE of tyl PKS. This led to the formation of 3-dihydronarbonolide (17), 3-dihydro-pikromycin (18) and 3-dihydro-narbomycin (19) (fig. 5C) (Yoon *et al.* 2002).

Yoon *et al.* performed more combinatorial approaches on pikromycin PKS. They swapped subunit 3 and 4 of pikromycin PKS for subunits 4 and 5 from tylosin PKS (fig. 6A). They also constructed a hybrid of subunit 3 with the ACP domain of pikromycin PKS and a KS, AT and KR domain of Tyl PKS subunit 4. The other hybrid subunit consisted of the KS and AT domains of pikromycin PKS subunit 4 and the KR, ACP and TE domains of Tyl PKS subunit 5 . These approaches led to compounds which were obtained earlier (fig. 6B) (Yoon *et al.* 2002).

The final approach Yoon *et al.* applied was the substitution of subunit 1 of pikromycin PKS with subunit 1 of Tyl PKS leading to the formation of 10-*epi*-10-deoxymethynolide (20), 12-*epi*-narbonolide (21), 10-*epi*-methymycin (22), 10-*epi*-neomethymycin (23), 12-*epi*-pikromycin (24) and 12-*epi*-narbomycin (25) (fig. 6C) (Yoon *et al.* 2002).

All the products in the study of Yoon *et al.* were the expected macrolactones, which were further post-PKS processed and led to bioactive macrolide structures. In most cases the native 10-deoxymethynolide (8) was produced also. This research showed that there is a large similarity between the pikromycin PKS and tyl PKS since they could produce the expected compounds. Although the yields were not as high as endogenous pikromycin PKS products it does indicate that the three-dimensional structure resembled the endogenous pikromycin PKS enough to carry out the native processing.

Another example is described by Kuhstoss 1996, they used the tyl PKS loading module (propionate specific) and positioned it in place of the platenolide I PKS loading module (acetate specific) which resulted in the production of 16-methylplatenolide (Kuhstoss *et al.* 1996).

So far these have been the only combinatorial approaches on tyl PKS. By comparison to DEBS this seems very minimal. But considering the little success the combinatorial derivatives of erythromycin had in becoming a clinical antibiotic one can think this is withholding the approaches on a less popular antibiotic like tylosin. It is easily said that all the approaches used on DEBS could be performed on tyl PKS given the modular organization of

both. Unfortunately, there is not a lot known on what unsuccessful combinatorial approaches have been applied to erythromycin. Knowing this would lead to a better understanding of how the PKSs work and in the comparison with tyl PKS would give more insight in future perspectives.

Challenges and future combinatorial approaches on tyl PKS

When we look at the modular organization of tyl PKS we can think that the organization in tyl PKS is more complicated because tyl PKS has 5 subunits (instead of 3 in DEBS) and therefore has more docking domains to consider. On the other hand this might be useful since the subunits can be swapped with other subunits of other PKSs type I, especially now that these domains are better understood, at least in DEBS (Buchholtz *et al.* 2009).

The combinatorial approaches that can be applied to tyl PKS seem therefore tremendous, since all the domains, modules and subunits could be exchanged with any of the examples used in DEBS and even more.

The biggest challenge is the three-dimensional structure of tyl PKS, which is not known to date. The best combinatorial approach therefore would be to use PKSs that resemble the tyl PKS largely but do have different properties (e.g. using other extender units, starter units or different reductive domains). This could be done by sequence analysis. In most research the linker and docking domains remain intact. This seems to be the best way to at least ensure that the domains, modules or subunits can interact. The three dimensional structure would hopefully remain comparable with the endogenous PKS and will give no further problems.

With these properties in mind we can think of a few examples of combinatorial approaches that can be applied to tyl PKS. The chain length can be altered by placing the TE domain after a preceding module. This should preferably be tried after module 2, 3, 5 or 6 since these are the last modules in a subunit and will mostly preserve the three dimensional structure. Hopefully this will lead to a 6, 8, 12 or 14 membered macrolactone, respectively. The TE module can be placed after module 1 and 4 as well. In DEBS, TE domains were placed within subunits which resulted in novel compounds, this could be applied to tyl PKS also (Rowe *et al.* 2001).

Although elongation of the PKS has not been applied very often it could be tried on tyl PKS. Because pikromycin PKS and tyl PKS resemble each other largely, we would

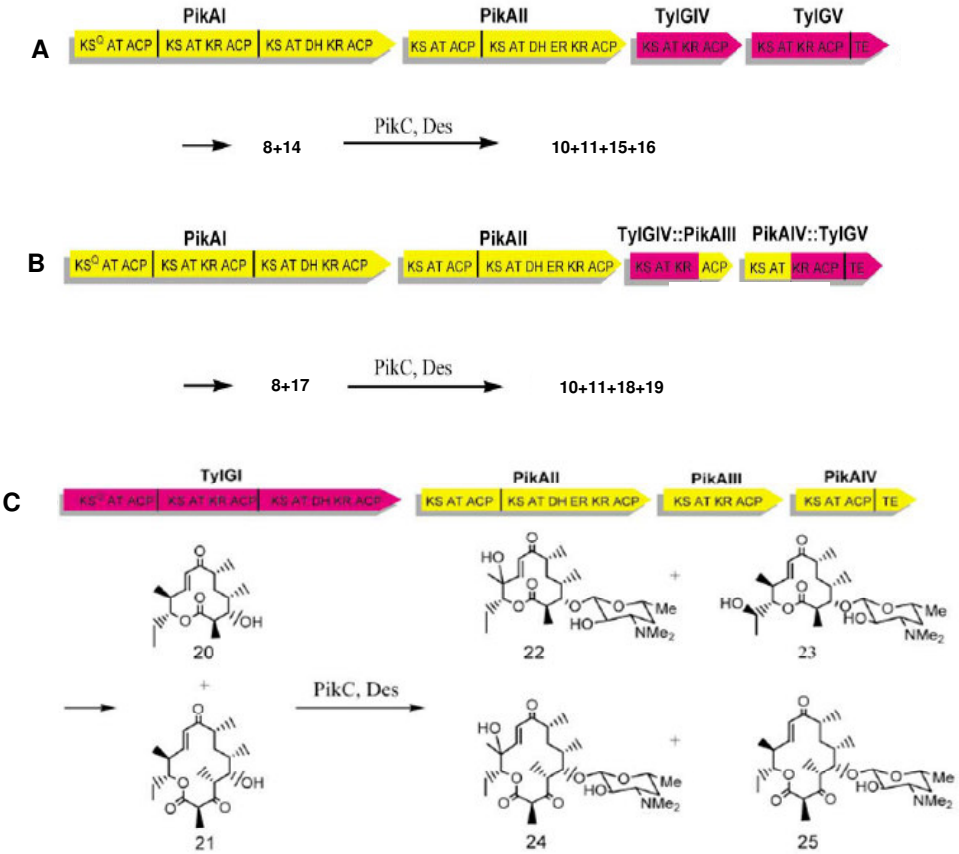

Figure 6. Additional combinatorial approaches on pikromycin PKS with segments of tylosin PKS as substituent.
Domains of pikromycin PKS are marked in yellow, domains of tyl PKS are marked in red.
Adapted from Yoon *et al.* 2002

suggest to place a module of pikromycin PKS into tyl PKS. Module 3 of pikromycin PKS seems to be the best candidate, because it contains the fewest domains and would therefore alter the three dimensional structure least. Because it has been shown that DEBS functioned with a module between module 1 and 2 this would be the first approach. In DEBS only little product was formed due to skipping, which we can expect to happen in this approach as well (Rowe *et al.* 2001).

When swapping domains, modules or subunits we can think of swapping a malonate specific AT domain of tyl PKS for a methylmalonate specific AT domain of DEBS. For example, the AT domain of module 1 of DEBS could be swapped for the AT domain of tyl PKS in module 1, which would probably lead to a methylated tylactone.

The same can be accounted for substituting a subunit. The resemblance of tyl PKS with pikromycin PKS suggests we can replace subunit 5 of tyl PKS with subunit 4 of pikromycin PKS. Subunit 4 of pikromycin PKS lacks a KR domain and uses methylmalonate instead of malonate as elongation unit. The expected product will have an increased oxidation state and will be methylated compared to the endogenous tylactone.

In DEBS the starter unit has been changed for the starter unit of avermectin which is 2-methylpropionate and 2-methylbutyrate instead of propionate in DEBS. This could be applied to tyl PKS since its starter unit is propionate as well. The expected compound will have an alternative starter unit.

All these combinatorial techniques will probably lead to the production of novel compounds. But considering that these approaches in DEBS did not lead to a novel antibiotic in clinical use, the usefulness of these approaches is questionable.

Now that docking domains and linkers are better understood more directed approaches are feasible then simply swapping domains, modules or subunits. One can think of the docking domain between DEBS2 and DEBS3 mentioned above with only a few residues active in the actual docking. When these are known for tyl PKS these could be altered when substituting subunits and substituting entire subunits (including docking domains) is then no longer the case. This is not part of combinatorial biosynthesis and is therefore not further discussed, for those interested a nice approach is described in Weissman 2006.

Discussion

A few examples of the combinatorial techniques applied to DEBS and the performed combinatorial approaches on tyl PKS have been described in this review. We have seen that chain lengths can be altered, domains, modules or subunits can be swapped, reductive states can be changed and starter units can be altered. Before comparing tyl PKS with DEBS we stated the challenges in combinatorial biosynthesis. We found that the organism producing an altered polyketide has to be well equipped, the domains have to have specialized features and specificities and the interdomain and intermodular linkers have to be considered. In addition to this, the docking domains must have the correct properties to fuse subunits and post-PKS processing must take place as expected. The clarification of the three dimensional structure of a PKS typ I will give insight in many of the processes that challenge combinatorial biosynthesis. Therefore further research is necessary.

We made a comparison of tyl PKS with DEBS and concluded that many of the approaches applied to DEBS can be applied to tyl PKS also. Therefore we tried to make suggestions towards combinatorial approaches that can be applied on tyl PKS. Hereby we took the challenges and successes of combinatorial techniques of type I PKSs into account. It is difficult to suggest novel approaches since most has been applied to DEBS. Almost all approaches amount to changing domains, modules or subunits which is what can be applied to other PKSs.

Considering the little success all the combinatorial approaches of DEBS had in creating a novel compound that made it to the clinic, we might think of a more rational approach then combinatorial biosynthesis. Therefore we need to investigate the mechanism of action of the macrolide antibiotics. Gaynor *et al.* show that there is a large amount of knowledge about these mechanisms (Gaynor *et al.* 2003). When these mechanisms and the way bacteria become resistant are fully understood we can alter the mechanism of action by rationally using combinatorial biosynthesis, resulting in a novel antibiotic compound. This inverted approach would hopefully lead to more bioactive compounds that will make it to the clinic.

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