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Rational Design of Polyketide Natural Products**

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Summary

Compounds of polyketide origin possess a wealth of pharmacological effects, including antibacterial, antifungal, antiparasitic, anticancer and immunosuppressive activities. Many of these compounds and their semi-synthetic derivatives are used today in the clinic. The first complete gene cluster encoding the polyketide antibiotic actinorhodin was cloned twenty years ago. The erythromycin gene cluster followed in 1990, and since then most of the gene clusters encoding commercially important drugs have also been cloned, sequenced and their biosynthetic mechanisms studied in great detail. Recent advances in the area of biosynthetic engineering of the enzymes involved in polyketide biosynthesis are presented in this review. The biosynthesis of a typical polyketide can be divided into three separate steps, including (*i*) choice of starter unit, (*ii*) the choice of extender units and the degree of beta-keto group reduction, and (*iii*) post-PKS tailoring of the basic polyketide backbone. Each of these steps represents a potential opportunity for the introduction of structural modification. The technologies to achieve this have now been highly developed and transferred into the industrial arena. The power of biosynthetic engineering will be exemplified by manipulations carried out on the erythromycin and the rapamycin biosynthetic gene clusters.

Key words: polyketide, biosynthetic engineering, erythromycin, rapamycin, antibiotic

Introduction

Polyketides are a large family of natural products with diverse chemical structures and a broad range of pharmacological activities. Among them many clinically important drugs can be found, such as the antibiotics erythromycin and tetracyclines, the immunosuppressants FK506 and rapamycin, cholesterol lowering lovastatin, the anticancer drugs daunorubicin and epothilone, and many other commercially important metabolites. Despite having such diverse structures and biological properties, the biosynthesis of these natural products follows a common mechanistic rule. Polyketide synthases (PKSs) are multi-enzyme complexes on which a polyketide chain is assembled by a stepwise condensation reaction of simple organic acids activated as coenzyme A (CoA) thioesters in a similar fashion to the chain elongation steps of classical fatty acid biosynthesis (*1,2*). Each elongation step may be followed by a different (but defined) level of reductive modification of the beta-keto group formed during the elongation step, before proceeding to the next round of chain extension. The complexity of the final structure is further enhanced by the use of different starter and chain extension units, variations in cyclisation of the nascent carbon chain backbone and a plethora of possible posttranslational modifications such as glycosylation, methylation, oxidation and others (*1,2*).

Following the cloning of the first, so-called reiterative PKS, actinorhodin PKS (*3*), and soon thereafter the first modular PKS for erythromycin biosynthesis (*4,5*), and the subsequent elucidation of the »genetic programming« of the PKS, a classification system was proposed based on the obvious similarity with fatty acid synthases (FASs). Modular type I PKSs are large (100–10 000 kDa) multi-modular enzymes with each catalytic site located on a separate protein domain, which are typically used only once (*1,2*).

Type II, aromatic PKSs, on the other hand, are multi-enzyme systems with each catalytic activity located on a separate protein subunit. The same catalytic centres are believed to be used again and again to extend the polyketide chain by iterative cycles. Each enzyme activity is encoded by a separate gene, leading to the classification of these enzymes as »type II PKSs«, through the parallel with fatty acid biosynthesis in *E. coli*, which is encoded by a similar set of genes (*1,2*). Knowledge of the genetic basis of programming of polyketide biosynthesis affords the opportunity to reprogram microbes to make novel structures. Compared to the modular type I PKS systems, aromatic PKSs, due to their reiterative biosynthetic mechanism, are far more difficult to reprogram. To date, it has been difficult to »reprogram« the chain length and the cyclisation pattern of type II PKS systems (*6–9*). However, a limited number of rational »designer rules« has been formulated in order to generate novel compounds (*10*).

Type I PKS system and its modular biosynthetic arrangement (and processive mechanism) offer, on the other hand, a wide range of opportunities for PKS reprogramming, which has been well documented in the literature. We will therefore limit the scope of this review to current advances of well-established and industrially applicable type I PKS engineering and the rational design of novel polyketide drugs.

Some Examples of the Reprogramming of Erythromycin and Rapamycin Polyketide Synthases

Medically important antibiotic erythromycin (Fig. 1) and the erythromycin PKS system from *Saccharopoly-*
spora erythraea were the first and, equally, the most attractive model systems for PKS manipulation. The PKS for the erythromycin precursor 6-deoxyerythronolide B (6-DEB) consists of three large (approx. 350 kDa each) polypeptides, each containing two extension modules. As well as the two extension modules, the N-terminal region of the first protein, 6-DEB synthase 1 (DEBS1), contains a loading module which comprises a di-domain consisting of an acyltransferase (AT) and an acyl carrier protein (ACP) domain. The loading module is involved in polyketide chain initiation and loads predominantly propionyl-CoA (Fig. 2).

Following the loading module, there are six extension modules, each containing at least three domains required to make one cycle of chain extension, beta-ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) domains as well as the optional beta-keto group reducing beta-ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains. The specific combination of present reductive domains corresponds to the degree of reduction that occurs within a specific round of chain elongation.

The heptaketide chain thus produced from a propionyl-CoA starter unit and six methylmalonyl-CoA extension units is finally released from the enzyme complex as the macrolactone 6-DEB by the action of a chain terminating thioesterase (TE) domain located at the C-terminal end of the last polypeptide DEBS3 (Fig. 2). The first enzyme-free intermediate 6-DEB then undergoes further post-PKS modifications, which include two hydroxylations, two glycosylations (addition of L-mycarose and D-desosamine), and *O*-methylation of the L-mycarosyl moiety (*1,2*).

Several approaches, mostly carried out during the 1990s by Cortés *et al.* (*4*) at the University of Cambridge and Donadio *et at.* (*5*) at Abbot Labs, resulted in novel structures. Modular design of type I PKSs gave rise to the idea of splicing domains and even modules of different PKS systems to provide recombinant enzymes capable of producing novel, »unnatural« polyketide natural products. A selected number of examples covering various approaches in the biosynthetic engineering will be discussed.
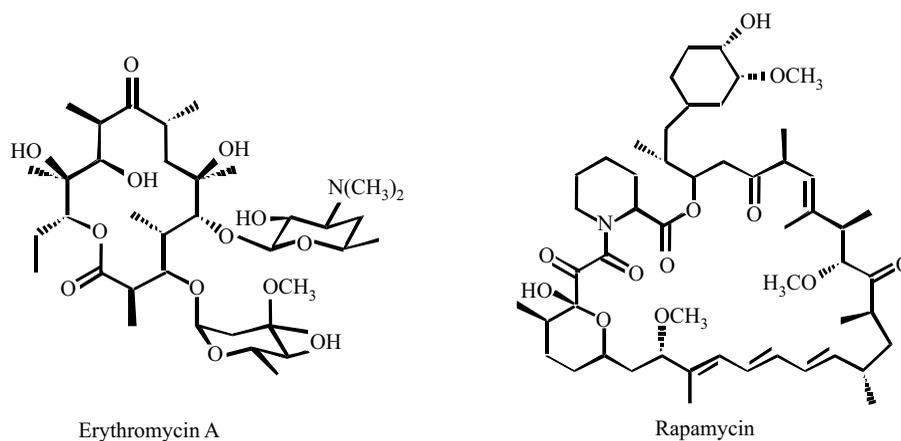

Erythromycin A   Rapamycin

Fig. 1. Structures of the antibacterial erythromycin A and the immunosuppressant rapamycin
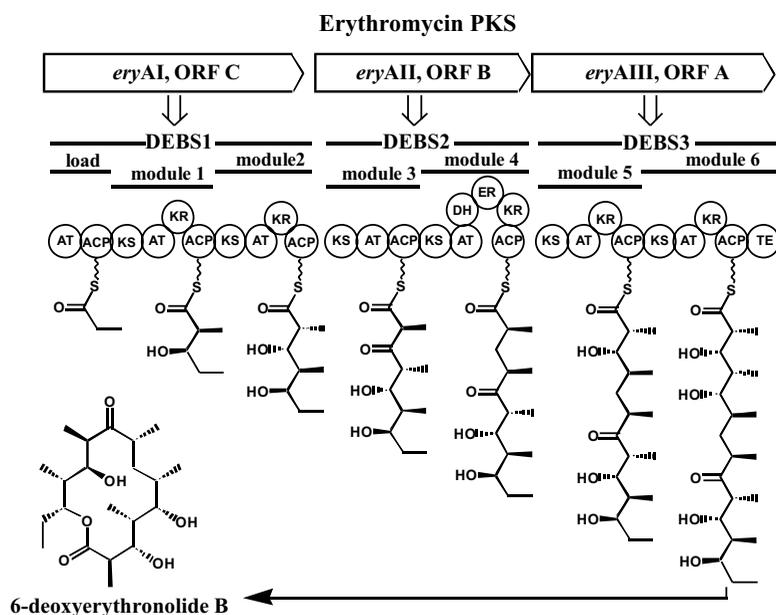
Erythromycin PKS

Fig. 2. Modular organisation of the 6-deoxyerythronolide B synthase (6-DEBS)

Structural Modifications of the Erythromycin Polyketide Backbone in the Area of the Starter Unit

There have been several successful approaches to introducing a structural modification(s) into the starter unit derived portion of the erythromycin polyketide backbone. One such technique based on precursor-directed biosynthesis was developed by Kosan scientists (*11*). This exploits the use of a PKS mutant in which one of the early-stage enzyme activities has been disabled, blocking the biosynthetic pathway. The biosynthesis of polyketides can then be restored by the introduction of an analogue of a natural biosynthetic intermediate. An example of such a strategy is the inactivation of the active site of the KS1 domain of DEBS1 by site-directed mutagenesis. Thus the natural substrate, an acyl thioester linked through the phosphopantetheine arm of the ACP domain of the preceeding module, cannot be transferred to the KS1 active site. A strain harbouring the mutant PKS was then fed with analogues of the diketide intermediate, activated as *N*-acetyl cysteamine thioesters (SNACs). Many of these were successfully transferred to the KS2 module, at which point chain elongation and reductive processing were once more initiated (*11*).

A second successful strategy involved replacing the loading module of a PKS with a heterologous loading module from a second PKS of a different starter acid specificity. This »loading module swap« was first successfully performed in the erythromycin PKS by replacing its loading module, which generally accepts propionyl-CoA, with the loading module from the avermectin--producing PKS of *Streptomyces avermitilis*. This PKS was shown to exhibit a very broad specificity for branched and cyclic carboxylic acids. When fed with various exogenous carboxylic acids a recombinant strain of *Saccharopolyspora erythraea*, containing the hybrid PKS, produced a number of novel bioactive erythromycin analogues derived from alternative starter units (*12*, Fig. 3).

Rapamycin Biosynthesis – Incorporation of Unnatural Starter Units

Rapamycin (Fig. 1) is a macrocyclic polyketide produced by *Streptomyces hygroscopicus* and has been shown to have immunosuppressive, antifungal and antitumor activities as well as displaying anti-inflammatory and neuro-regeneration properties. Due to this broad biological activity, rapamycin has generated a great deal of interest in the scientific community, and this has induced a significant effort to determine the genetic and biochemical mechanisms of its biosynthesis. In 1995 the rapamycin biosynthetic gene cluster was cloned and the sequence of the ~110 kb rapamycin biosynthetic cluster determined (*13*).

Rapamycin biosynthesis occurs via a mixed type I PKS and non-ribosomal peptide synthetase (NRPS) system (*13,14*). Rapamycin biosynthesis begins with an unusual shikimate-derived starter unit onto which 7 methylmalonyl-CoA and 7 malonyl-CoA extension units are added during the chain elongation process. Macrocycle formation is not catalysed by a TE domain, but through incorporation of the amino acid L-pipecolic acid by an NRPS like protein, which also forms ester and amide bonds, to close the macrocyclic structure (Figs. 1 and 4).

As mentioned earlier, the PKS uses a shikimate-derived 4,5-dihydroxycyclohex-1-enecarboxylic acid (DHCHC) starter unit (*15*). Selection and activation of the rapamycin PKS carboxylic acid starter unit is carried out by a carboxylic acid-ligase (CL) like domain (part of the PKS loading module) in a manner analogous to that of the adenylation domains of NRPS systems. Efficient incorporation of the *pseudo*-starter acid 1,2-dihydro-DHCHC during stable isotope feeding experiments indicated that the domains of the PKS involved in starter unit selection may have broad substrate tolerance (*15*). With this in mind, Lowden *et al.* (*16*) carried out feeding experiments in which a number of exogenous, potential starter acids were fed to a culture of wild type *S. hygroscopicus*, thus
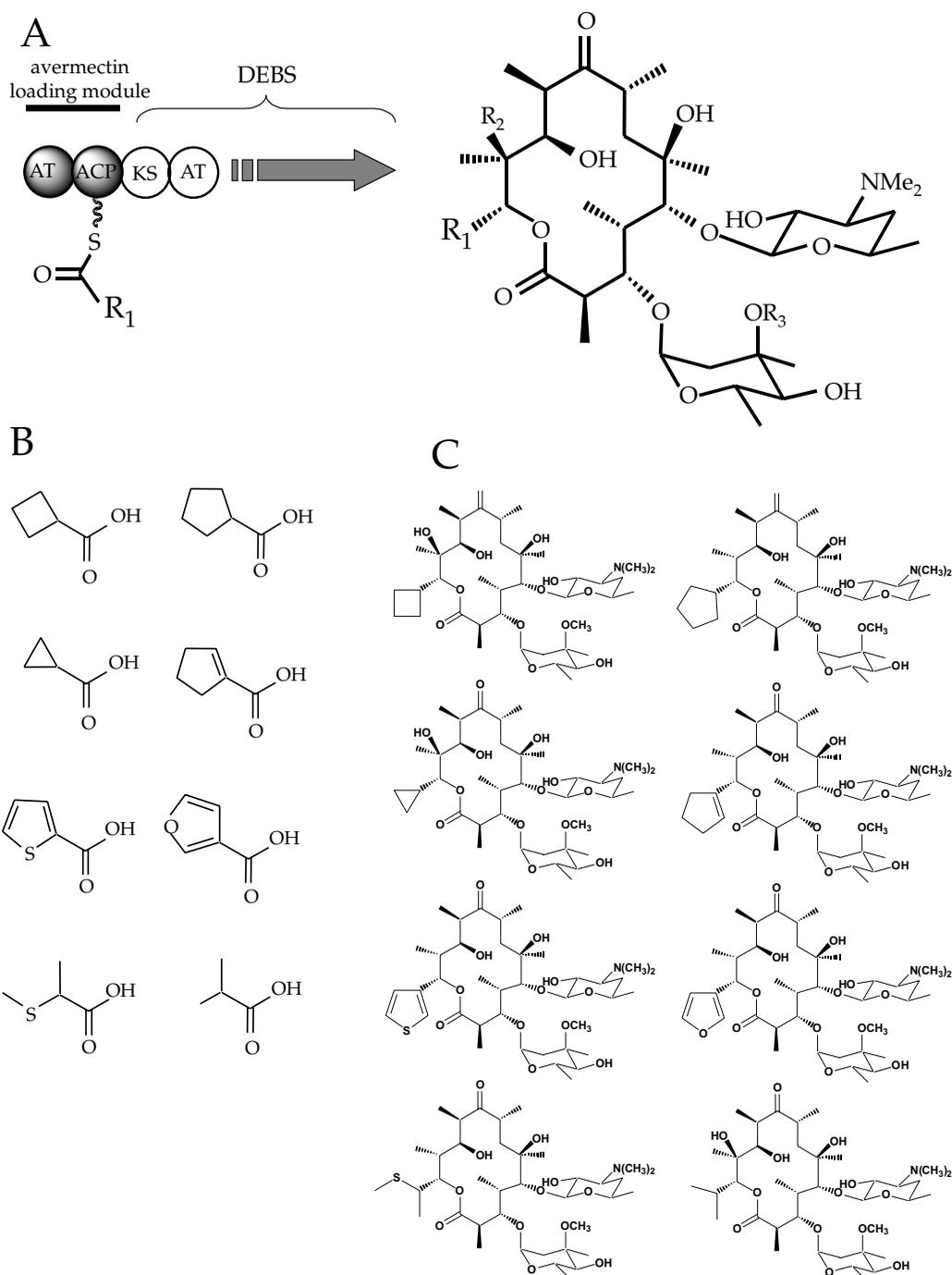
Fig. 3. Hybrid modular PKS containing the avermectin PKS loading module attached to DEBS PKS (**A**). The exogenous carboxylic acids fed to the recombinant strain of *Saccharopolyspora erythraea* (**B**) and a number of novel erythromycin analogues (**C**)

producing a number of novel rapamycin derivatives generated by precursor-directed biosynthesis. The cyclohexane, cycloheptane and cyclohexene carboxylic acids fed to cultures of *S. hygroscopicus* yielded from 25 to almost 50 % of novel rapamycin analogues in addition to the natural rapamycin, demonstrating substrate flexibility of the loading and extension modules of the rapamycin PKS. These novel compounds were also substrates for the post-PKS steps of rapamycin biosynthesis (*16*, Fig. 5).

Polyketide Chain Extension – Modification of the Polyketide Backbone

A strategy similar to that of loading module swapping can be applied to chain extension AT domain. The AT domain is responsible for selecting the extension unit to be incorporated into the polyketide chain, and can thus be swapped with a heterologous AT domain with an alternative substrate specificity in order to incorporate a different extension unit at the chosen posi-
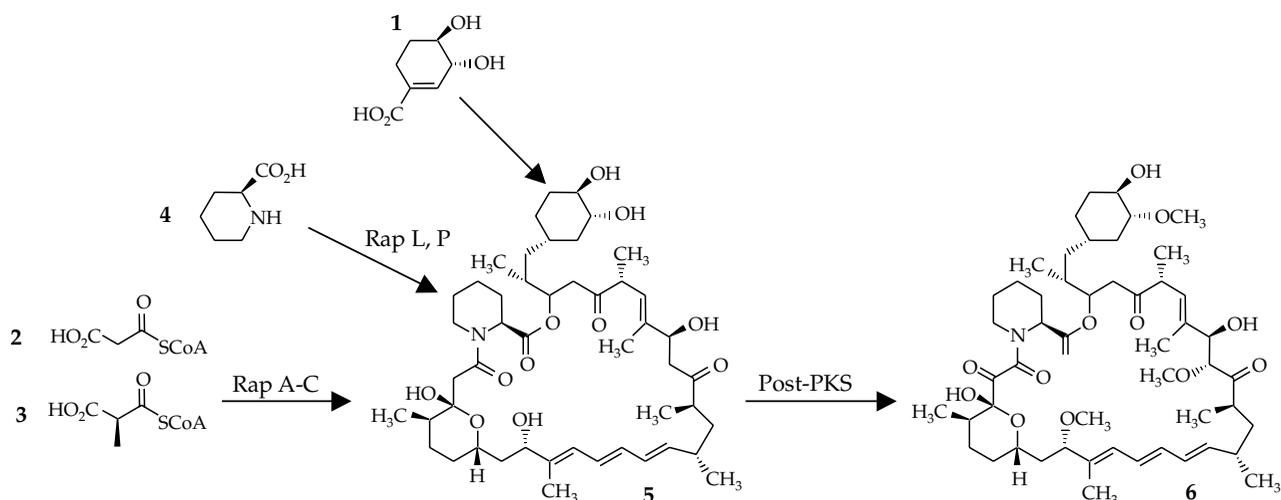
Fig. 4. Biosynthesis of rapamycin (**6**). The first macrocyclic intermediate pre-rapamycin (**5**) and the precursors of pre-rapamycin, dihydroxycyclohex-1-enecarboxylic acid (**1**), malonyl-CoA (**2**), methylmalonyl-CoA (**3**) and L-pipecolic acid (**4**)
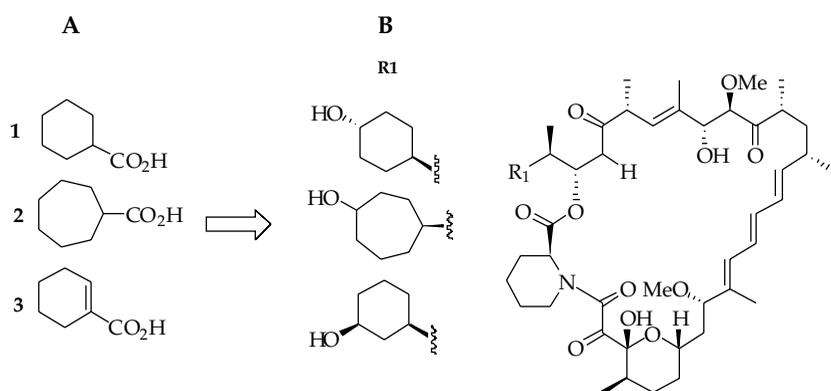

Fig. 5. Cyclohexanecarboxylic acid (**A1**), cycloheptanecarboxylic acid (**A2**), and cyclohex-1-enecarboxylic acid (**A3**) fed to the cultures of *Streptomyces hygroscopicus* and the rapamycin analogues produced by feeding alternative starter units (**B**)

tion. Many such AT domain swaps have been successfully performed (*17,18*). The final AT swap experiment in the erythromycin PKS, in which the methylmalonyl-
-CoA specific AT domain of module 4 is replaced with the malonyl-CoA specific rapamycin AT domain from the rapamycin PKS module 2, has recently been achieved (*19*). The resulting strain produced 6-desmethylerythromycin D as the predominant product. This AT domain swap completes the library of malonyl-CoA AT swaps for the erythromycin PKS (Fig. 6).

A different approach to this »standard« AT domain swap was reported by two different groups (*20,21*). Conserved amino-acid motifs believed to be crucial for substrate selection were modified by site-directed mutagenesis in order to change the specificity of an AT domain from methylmalonyl-CoA to malonyl-CoA. However, the specificity of the AT domain was not exchanged entirely, and resulted in the formation of products derived from both methylmalonyl-CoA and malonyl-
-CoA as extender units (*20,21*).

Alteration of the Oxidation Level of a Polyketide Backbone

The oxidation level of the beta-keto group of an extension unit can be modified by altering the composition of the reductive loop domain(s). This can be achieved by simple loss-of-function (deletions) or gain-of-
-function (insertions) mutations of the KR, DH and ER domains. Donadio *et al.* (*5*) at Abbot first demonstrated this kind of modification by inactivation of the KR domain of module 5 of the erythromycin PKS (*22*). The resulting recombinant strain of *Saccharopolyspora erythraea* resulted in a 6-DEB derivative in which the hydroxyl-
-group at C5 was replaced with a keto group (*22*).

Modification of the Post-PKS Tailoring of Polyketides

Biological activity of polyketide antibiotics is often dependent on the post-PKS tailoring of the initial carbon
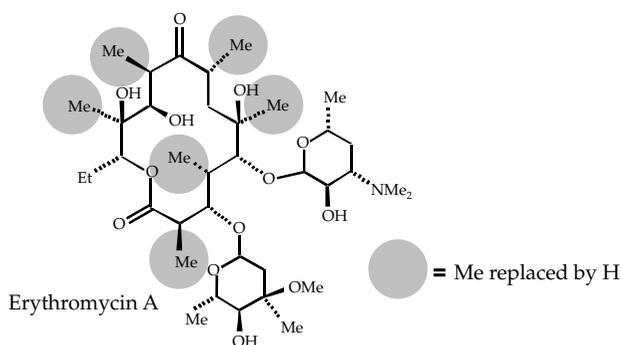
Fig. 6. Successful acyltransferase (AT) domain swap experiments in the erythromycin polyketide synthase (PKS) in which malonyl-CoA was incorporated instead of the methylmalonyl-CoA extender unit

skeleton produced by the PKS. Polyketide backbones can be further modified by cytochrome P450 monooxygenases, methylated by methyl transferases or have sugars attached through the action of glycosyl transferases. For example the sugar moieties appended to many polyketides play key roles in the molecular recognition between the antibiotic and its cellular target (*23–25*).

Manipulation of the late steps of the rapamycin biosynthesis is an illustrative example of the power of post-PKS engineering. Kosan scientists generated 16-O-desmethyl-27-desmethoxyrapamycin using a knock-out strain of *S. hygroscopicus* (*26*). Biotica scientists reported the formation of the first PKS-free intermediate of rapamycin biosynthesis (pre-rapamycin) using a recombinant strain of *S. hygroscopicus* in which the entire region of the rapamycin gene cluster thought to carry the genes responsible for the post-PKS steps was deleted (*27*, Fig. 4). The resulting strain was then used to generate a broad spectrum of rapamycin analogues with various degrees of post-PKS processing (*28,29*).

Discussion

In addition to utilizing conventional chemical approaches, the well-established modularity of the type I PKS multi-enzyme complexes readily lends itself to the use of genetic engineering to develop non-natural analogues of commercially valuable products. Not only can a single modification be introduced into a polyketide structure, but even multiple alterations can be incorporated simultaneously through the rational approach of PKS engineering. Furthermore, by varying the degree of post-PKS processing, and in combination with the incorporation of various »unnatural« starters, it is now possible to produce a large number of potentially useful unnatural polyketide compounds with multiple modifications in the chemical structure (*18,28*).

The technology of biosynthetic engineering of enzymes involved in polyketide synthesis has also disadvantages compared to the semi-synthetic chemistry approach. Although it may sound very lucrative to be able to replace various functional groups of a natural polyketide, it must be noted that not all of the modifications

attempted have been successful. This may be due to many reasons such as incompatibility of the particular domains used and usage of unsuitable protein/domain splice sites. Often, the modified polyketide intermediate is not the most suitable substrate for the downstream enzyme activities both within the PKS and in the post-PKS processing steps (*19*). Incorrect polyketide formation (processing) can also arise from the lack of appropriate substrate supply of extension units in a heterologous host (*30*).

However, the technology does very often give rise to the target product. It has been clearly demonstrated that the biosynthetic engineering of PKS can be productive and it is a proven tool in the drug-discovery process. It represents a very rational route for the introduction of chemical modifications to the medically important drugs, which often cannot be achieved by synthetic chemistry.

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Biosinteza poliketidnih prirodnih spojeva

Sažetak

Spojevi poliketidnog izvora imaju širok spektar bioloških aktivnosti, kao što su antibakterijsko, antifungalno, antikancerogeno i imunosupresijsko djelovanje. Danas se veliki broj tih spojeva i njihovih polusintetskih derivata koristi u medicini. Geni koji kodiraju biosintezu antibiotika aktinorodina klonirani su prije dvadest godina i čine prvu kloniranu cjelovitu gensku nakupinu za biosintezu poliketidnog antibiotika. Ubrzo nakon toga, godine 1990. klonirana je i cjelovita nakupina gena za biosintezu antibiotika eritromicina. U posljednjem su desetljeću klonirane i sekvencionirane gotovo sve genske nakupine komercijalno važnih biološki aktivnih poliketida, a njihovi putovi biosinteze proučavani su vrlo podrobno. Razvoj tehnologije biosintetskog inženjerstva enzima koji kataliziraju biosintezu poliketida glavna je tema ovog revijalnog rada. Put biosinteze tipične poliketidne molekule može se podijeliti u tri osnovna koraka, koji obuhvaćaju (*i*) izbor početne jedinice, (*ii*) izbor produžne jedinice i stupanj redukcije β-keto skupine poliketidnoga lanca i (*iii*) modifikacija strukture dovršenoga poliketidnog kostura. Svaki od tih koraka u procesu bio-

sinteze omogućuje unošenje promjena u strukturu poliketidnoga spoja. Tehnologija koja omogućuje takve manipulacije danas je vrlo dobro razvijena i vrlo često primjenjivana u industriji. Biosinteza u medicini važnih poliketidnih spojeva, kao što su antibiotik eritromicin i imunosupresor rapamicin, te generiranje novih biološki aktivnih derivata, odličan su primjer primjene tehnologije biosintetskog inženjerstva.