S4-4

Combinatorial Biosynthetic Strategies for Generation of Hybrid Polyketides

Sang Kil Lee[1], Jay Sung Joong Hong[1], Won Seok Jung[1], and Yeo Joon Yoon[2]

[1]School of Chemical Engineering, Seoul National University, Seoul, Korea; [2]Division of Nano Science and Department of Chemistry, Ewha Womans University, Seoul, Korea

1. Generation of novel polyketides by modifying polyketide synthases (PKSs)

Polyketides are a large class of natural products that possess diverse structures and pharmacological activities (Katz, 1997). The biosynthetic pathways of macrolide polyketides involve the formation of macrolactones by the activity of the large multifunctional enzymes, modular polyketide synthases (PKSs), followed by specific post-PKS modifying steps such as oxidation, methylation, acylation, and glycosylation. The pikromycin (Pik) polyketide synthases (PKSs) of *S.venezuelae* are comparatively suitable for combinatorial biosynthesis because they can simultaneously produce 12- and 14-membered ring macrolides (Xue *et al.*, 2000). The unusual nature of the *S.venezuelae* system provides a potentially useful tool for generating multiple products from a single hybrid modular PKS by combinatorial biosynthesis. Modification of the pikromycin biosynthetic gene cluster from *S.venezuelae* have allowed the generation of several novel polyketides antibiotics (Yoon *et al.*, 2002).

1-1. Generation of multiple bioactive macrolides by modifying pikromycin PKS

The plasmid-based replacement of the multifunctional protein subunits of the pikromycin PKS (Fig. 1) in *S.venezuelae* by the corresponding subunits from heterologuous modular PKSs resulted in recombinant strains that produce both 12- and 14-membered ring macrolactones with predicted structural alterations. Complementation of the *pikAI* deletion mutant by the corresponding enzyme TylGI expressed on a plasmid resulted in producing macrolactones of molecular weight identical to those from wild-type *S.venezuelae*. Polyketides with novel structures can be also generated through complementation of PikAIV with native and several hybrid TylGVs and expression of hybrid TylGIV and TylGV in the corresponding pikAIII and pikAIV deletion mutant. Biological activity of novel polyketides produced by modifying pikromycin PKS can be assayed by TLC separation of the culture extract. This work demonstrates that Pik PKS in *S.venezuelae* is amenable to genetically modification and hybrid pik PKS can produce multiple bioactive macrolides that differ in size of the macrolactone core.

1-2. Heterologous expression of the tylactone gene cluster and production of a novel polyketide

The gene cluster responsible for tylactone biosynthesis in *Streptomyces fradiae* (fig. 2) was completely

expressed in a heterologous host, *Streptomyces venezuelae*. As a result, a 16-membered macrolactone, tylactone and a novel 14-membered macrolactone were produced in this system. In order to express the tylactone gene cluster and produce tylactone, we constructed the engineered mutant of *S.venezuelae*, in which the polyketide synthase (PKS) related to biosynthesis of the natural polyketides is deleted, and used the two-vector system. The availability of a heterologous expression system could be applied to several important areas. First, this system is likely to be accessible in enhancing productivity of polyketides from their authentic host having low productivity. Second, it should be possible to construct an expression system producing novel polyketides with the post-PKS biosynthetic tailoring genes, such as hydroxylase and glycosyltransferase. A 14-membered macrolactone could be produced in this study due to the unusual property of *S. venezuelae*. Naturally, *S.venezuelae* produces both the 12- and 14-membered macrolactones by a mechanism yet to be elucidated.

This study is a rare example presenting the possibility of expressing the entire PKS in a heterologous host and producing the structurally diverse polyketides.

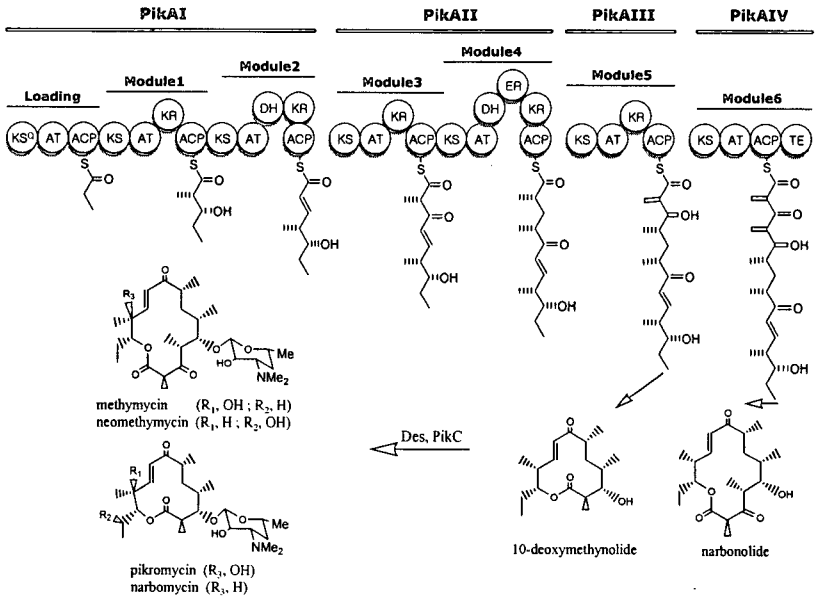

Fig. 1. Modular organization and the products of pikromycin PKS.

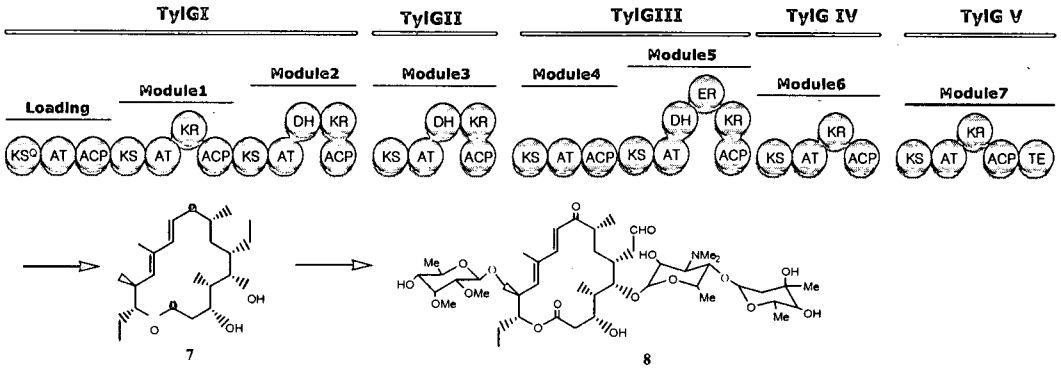

Fig. 2. Modular organization and the products of tylosin PKS.

2. Production of novel polyketides by post-PKS modifying step

The biosynthetic pathways of macrolide antibiotics produced by actinomycetes involve the formation of macrolactones by modular type I polyketide synthase (PKS) in the first stage, followed by specific post-PKS modifying steps such as oxidation, methylation, acylation and glycosylation. The structural diversity of PKS products is further increased by these modification reactions. As these modifications are also often critical for biological activity, a current challenge for combinatorial biosynthesis is to develop approaches that lead not only to novel macrolactones but also to ones that provide fully elaborated structures generated by post-PKS tailoring enzymes.

2-1. Hydroxylation or other oxidative steps

One of the post-PKS modification reactions, hydroxylation or other oxidative steps catalyzed by cytochrome P450 monooxygenases are often key to the structural diversity and biological activity of macrolide antibiotics. Therefore, the identification and investigation of the substrate-flexibilities of P450s is significant to the generation of a variety of unnatural polyketides through combinatorial biosynthesis.

The substrate-flexibilities of several cytochrome P450 monooxygenases involved in macrolide biosynthesis were investigated to test their potential for the generation of novel macrolides. PikC hydroxylase (Fig. 3A) (Xue *et al.*, 1998) in the pikromycin producer *S. venezuelae* accepted oleandomycin as an alternative substrate and introduced hydroxyl group at the C-4 position, which is different from its intrinsic C-12 hydroxylation position in the natural substrates (Fig. 4A). This is the first example of C-4 hydroxylation activity for 14-membered macrolides. EryF hydroxylase (Fig. 3B) (Andersen *et al.*, 1992) from erythromycin pathway and OleP oxidase (Fig. 3C) (Shah *et al.*, 2000) from oleandomycin pathway also showed a certain degree of plasticity towards alternative substrates (Fig. 4B, C). We have shown that EryK (Fig. 3D) (Stassi *et al.*, 1993) can accept the substrates of PikC from *S. venezuelae* which is responsible for the hydroxylation of YC-17 and narbomycin. In a *S. venezuelae pikC* deletion mutant, EryK could catalyze the hydroxylation of YC-17 and narbomycin to methymycin/neomethymycin and pikromycin, respectively (Fig. 4D).

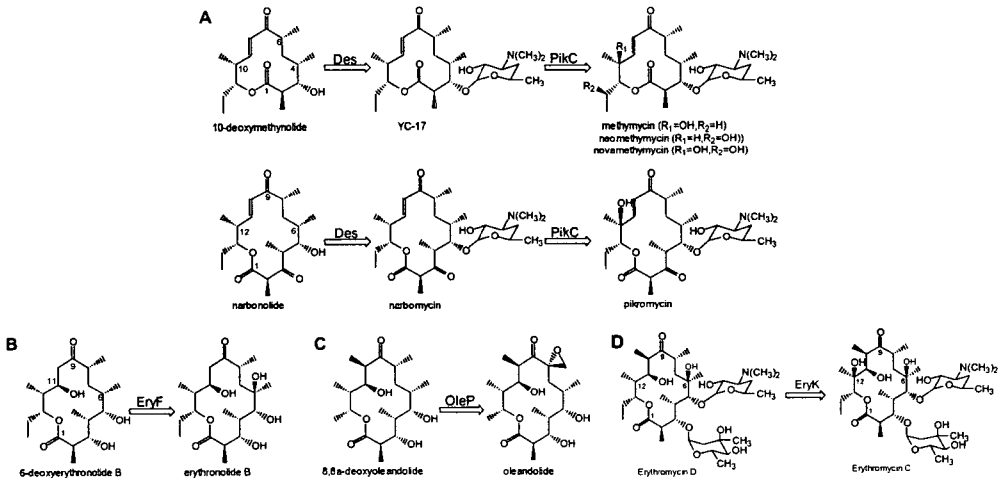

Fig. 3. The natural functions of hydroxylases used in this study.

These results demonstrate the high potential usefulness of these enzymes to diversify macrolactones by post-PKS oxidations.

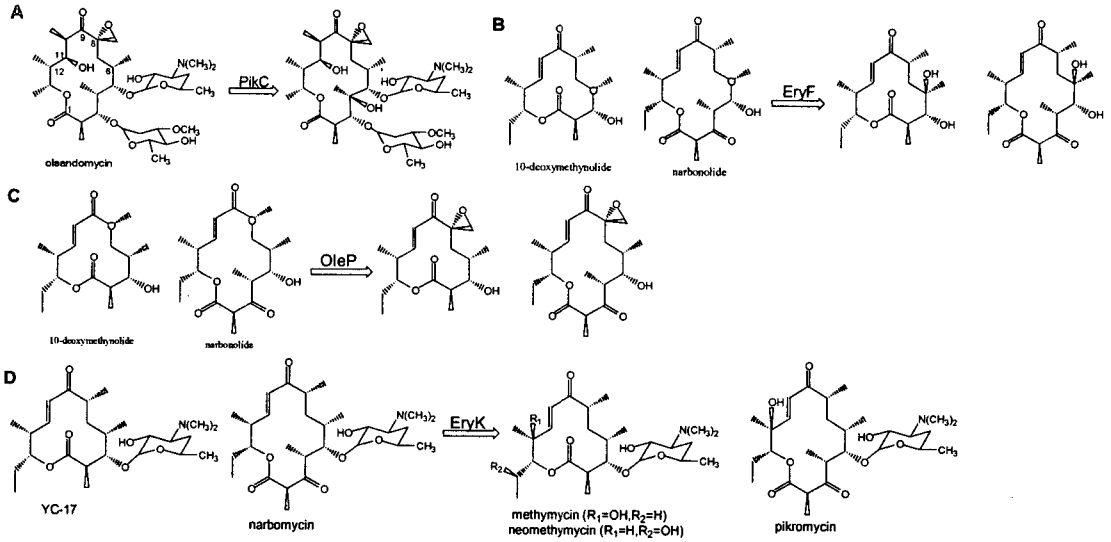

Fig. 4. Unnatural substrates used in this study and produced novel polyketides.

2-2. Glycosylation

Combinatorial biosynthesis of the deoxysugar moiety of macrolide antibiotics is a promising method for modifying natural products from microorganisms for development of novel drugs. As deoxysugars are essential components of macrolide antibiotics, there is considerable interest in the possibility of using combinatorial biosynthesis to generate macrolactones attached with new deoxysugars. The sugar component was found to be involved in molecular recognition between the antibiotic and its cellular target (Weymouth-Wilson, 1997; Kirschning *et al.*, 1997). Given the central role of sugars in determining macrolide activity profiles, pathways of deoxyhexose biosynthesis offer attractive targets for manipulation in the development of novel macrolide derivatives. In the biosynthesis of methymycin/pikromycin, an amino-sugar D-desosamine is attached to each of the aglycones (Fig. 5). Eight open reading frames (*desI-desVIII*) are suggested to be involved in desosamine biosynthesis. In a series of gene inactivation experiments (Borisova *et al.*, 1999; Zhao *et al.*, 1998a,b, 2001), the biosynthetic pathway for D-desosamine was proposed. Each gene has been assigned a function except for *desVIII* that remains to be further investigated. These studies also revealed that DesVII could accommodate the intermediate sugars in the desosamine pathway to be accepted as substrates for incorporation into the macrolactones. An approach to attach a non-native sugar by recombination of heterologous sugar genes, *strM* and strL from *Streptomyces griseus*, have shown that DesVII is substrate tolerant towards the deoxysugar, L-rhamnose (Yamase *et al.*, 2000).

To further investigate the feasibility of attaching non-native sugars, a mutant strain of *Streptomyces venezuelae* was genetically engineered for the biosynthesis of two different macrolactones (10-deoxymethynolide and narbonolide) and a key intermediate deoxysugar (4-keto-6-deoxy-D-glucose) of macrolide antibiotics. The 'sugar flexible' glycosyltransferase, desVII has recently been shown to transfer

4-keto-6-deoxy-D-glucose to 10-deoxymethynolide which is converted to D-quinovose by a pathway-independent reductase. In accordance with this finding, transformation by a plasmid encoding genes involved in glycosylation (*desVII* and *desVIII*) lead to the production of two D-quinovose glycosylated macrolides thus validating the system (Fig. 5). Heterologous sugar genes can be introduced into this mutant strain in order, test the substrate tolerance of DesVII and to biosynthesize novel methymycin/pikromycin derivatives.This system has been used to demonstrate that an intermediate sugar (D-olivose) from the oleandomycin deoxysugar pathway can be attached to both macrolactones. The deoxysugar genes including, *oleV* and *oleW* from *Streptomyces antibioticus* and *urdR* from *Streptomyces fradiae*, required for the biosynthesis of D-olivose were introduced into this mutant to produce the expected olivosyl methymycin/pikromycin analogs.In addition, both D-quinovosyl and D-olivosyl derivatives were further modified through the action of a hydroxylase (PikC) present in the system hence generating even greater structural diversity. This system is useful in creating multiple novel compounds by combinatorial biosynthesis and furthermore these results have provided new insights into the glycosylation process in *S. venezuelae*.

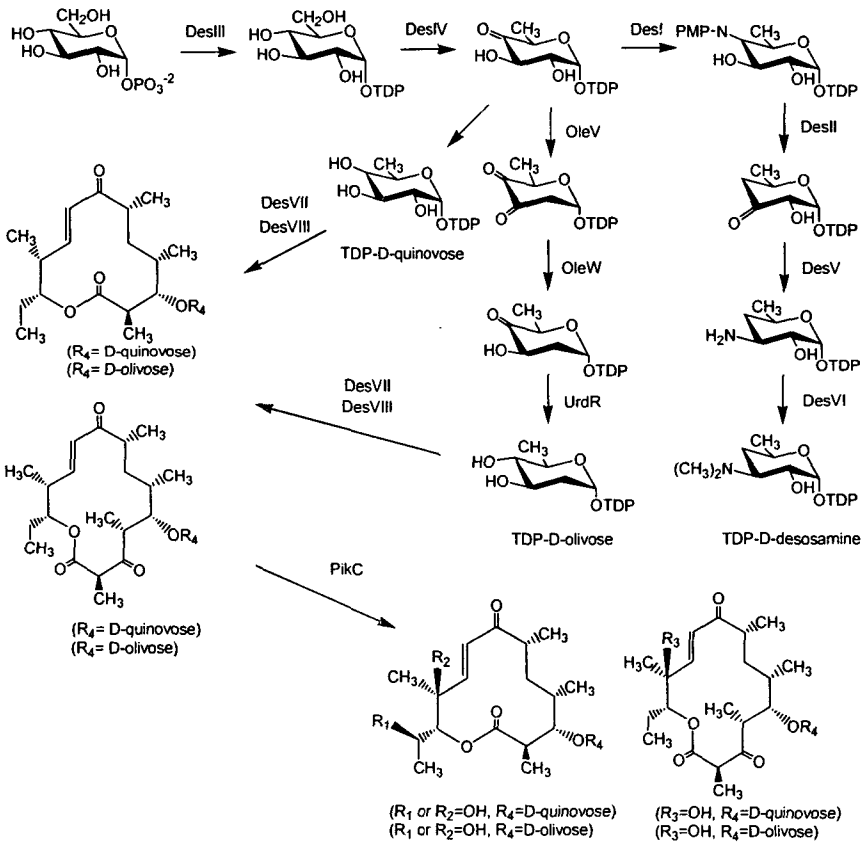

Fig. 5. Proposed pathways for the biosynthesis of TDP-D-desosamine, TDP-D-quinovose and TDP-D-olivose. The structures of the hybrid macrolides synthesized are also indicated.

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