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Short communication

Combinatorial biosynthesis of 5-*O*-desosaminyl erythronolide A as a potent precursor of ketolide antibiotics

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ABSTRACT

Ketolides, characterized by possessing a 3-keto group in place of the L-cladinose moiety of erythromycin A, are the recent generation of antimicrobials derived semi-synthetically from the 14-membered ring macrolide erythromycin A. The multi-step synthetic route to ketolides can be shortened by using 5-*O*-desosaminyl erythronolide A as a precursor, which reduces the steps for the removal of L-cladinose attached at the C-3 position in erythromycin A. Deletion of an *eryBV* gene encoding mycarosyl glycosyltransferase in the erythromycin-producer *Saccharopolyspora erythraea* resulted in the accumulation of 5-*O*-desosaminyl erythronolide B. *In vivo* expression of the cytochrome P450 gene *pikC*, which encodes the substrate-flexible hydroxylase from the pikromycin biosynthetic pathway of *Streptomyces venezuelae*, in the *eryBV* deletion mutant strain of *Sac. erythraea* led to 5-*O*-desosaminyl erythronolide A production.

Erythromycin A (ErA, **1**), which consists of two appendages sugar moieties, is a well-known and clinically important 14-membered ring macrolide antibiotic (Fig. 1). Ketolides, the most recent generation of antimicrobials derived from ErA (**1**), differ from macrolides as having a 3-keto functional group instead of an L-cladinose moiety and *N*-substituted carbamate extension at C-11 or C-12 positions in erythromycin. The synthesis of ketolides begins by hydrolysis of L-cladinose sugar from 6-*O*-methyl erythromycin A which is chemically modified from natural ErA (Zhanel et al., 2002). *Saccharopolyspora erythraea* NRRL2338 is known to produce ErA, and its biosynthetic gene cluster and pathway have been reviewed (Staunton and Weissman, 2001). In the biosynthetic pathway to ErA (**1**), two EryBV and EryCIII glycosyltransferases are responsible for attachment of L-mycarose and D-desosamine onto the corresponding substrates erythronolide B and 3-*O*-mycarosyl erythronolide B, respectively (Fig. 1). Furthermore, the post-PKS tailoring enzymes in the pikromycin biosynthetic gene cluster of *Streptomyces venezuelae* are valuable for combinatorial biosynthesis of macrolide derivatives, in particular the ability of PikC to hydroxylate a range of macrolides in diverse patterns have been exploited (Lee et al., 2006a,b; Xue et al., 1998a,b). The aim of this study is to generate 3-decladinosyl derivative of ErA as a potent precursor for ketolide synthesis directly from erythromycin-producing strain *via* engineering biosynthetic pathway and/or combinatorial biosynthesis. This can simplify the current multi-step synthetic process. Here, we describe the construction of *eryBV* deletion mutant *Sac. erythraea* YJ584, producing 5-*O*-desosaminyl erythronolide B (DesEB, **2**) and the biosynthesis of 5-*O*-desosaminyl erythronolide A (DesEA, **3**), a hydroxylated form of DesEB (**2**), by *in vivo* expression of PikC from *S. venezuelae* in *Sac. erythraea* YJ584 mutant strain.

The *eryBV* deletion plasmid (pYJ584) was constructed by introducing PCR-amplified fragments from the genomic DNA of *Sac. erythraea* NRRL2338 [provided by J.M. Weber (Fermalogic Inc., USA)], flanking on either side of e*ryBV* using the LA5F/LA5R and RA5F/RA5R primer pairs (Tables 1 and 2), into pWHM3 (Vara et al., 1989). This plasmid was then transformed into *Sac. erythraea* NRRL2338 protoplasts (Weber and Losick, 1988). Double crossovers yielding targeted gene deletion were selected for thiostrepton (25 $\mu g\,ml^{-1}$) sensitive phenotype, and the mutant's genotype was confirmed by Southern hybridization of genomic DNA (Fig. 2). The disappearance of ErA (**1**) and erythromycin B (ErB, **4**) commonly detected in wild-type strain, and the appearance of DesEB (**2**) as the major macrolide ($\sim$580 $\mu g\,l^{-1}$) was confirmed by HPLC-ESI-MS/MS analyses of organic extracts of *Sac. erythraea* YJ584 culture (Fig. 3A and B).

Plasmids pYJ591 and pYJ586, for expression of EryBV and PikC, respectively, were separately constructed in pWHM3 derivatives (Table 2). The plasmid pYJ585 was made by introducing the DNA fragment containing *actI* promoter, which was PCR-amplified from pHGF7505 (Yu et al., 2001) using the actIF/actIR primer pair, into pWHM3. The plasmid pYJ00 was also constructed by introducing a DNA fragment containing *erm*E-distal part (homologous region for plasmid-mediated recombination), which was PCR-amplified from the genomic DNA of *Sac. erythraea* using primers disF/disR,

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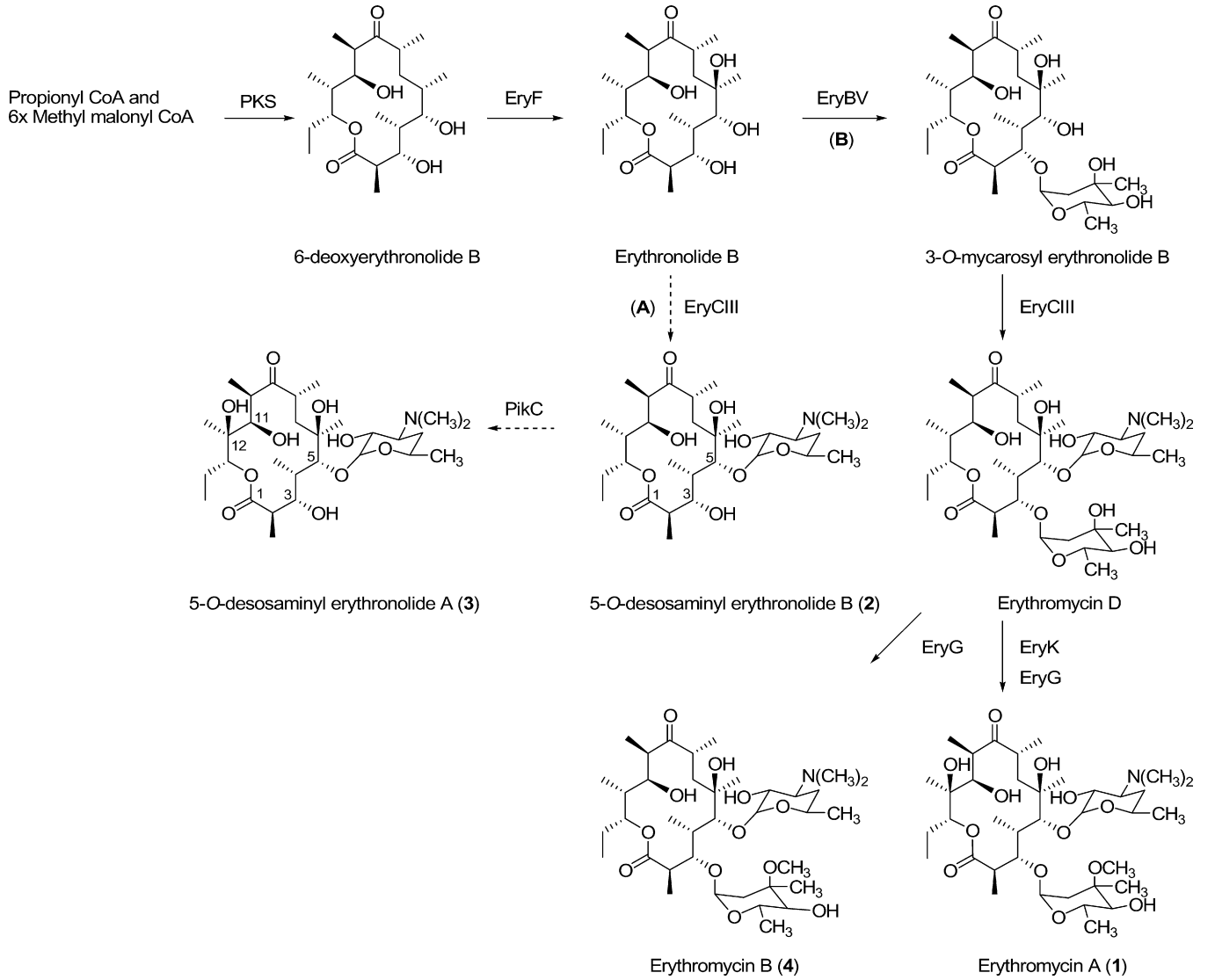

Fig. 1. Biosynthetic pathways to erythromycin A and its derivatives. (A) Natural biosynthetic pathway to erythromycin A (**1**) (solid arrows). (B) Biosynthesis of 5-*O*-desosaminyl erythronolide B (**2**) and 5-*O*-desosaminyl erythronolide A (**3**) in *Sac. erythraea* YJ584/pYJ586 (dashed arrows). Erythromycin B (**4**) was detected in wild type as shunt product.

into Litmus28 (Tables 1 and 2). The fragment containing *pikC* amplified from the genomic DNA of *S. venezuelae* by PCR using primers pikCF/pikCR was ligated into corresponding sites in pYJ00, and then the DNA fragment including *pikC* and *erm*E-distal part was cloned into pYJ585 designating pYJ586. Similarly, the PCR-amplified fragment containing *eryBV* from the genomic DNA of *Sac. erythraea* using primers eryBVF/eryBVR was ligated into pYJ00, and then the DNA fragment including *eryBV* and *erm*E-distal part was cloned into pYJ585 designating pYJ591 (Tables 1 and 2). The resulting plasmids were transformed separately into *Sac. erythraea* YJ584 protoplasts, and chromosomal integration of each plasmid was confirmed by Southern hybridization (data not shown). Self-

Table 1
List of deoxyoligonucleotide primers used in this study

Primers	Primers sequence (5′–3′)	Restriction sites
disF	CGAGACGAATTCGGCGGAATATTAACGGTTAAA	EcoRI
disR	GATGTTATGCATCGAATGCACGACGAAGAACTG	NsiII
LA5F	GCCCGTGGATCCGACCTGGAAAGCGAGCAA	BamHI
LA5R	ACCCGCACTAGTGCTCCTCGGTGGGGGTCA	SpeI
RA5F	GCCACTAGCGGTTTCCGACCGACA	NheI
RA5R	TCTGCGTCTAGACCGGAATCGTCCGGTCGC	XbaI
eryBVF	ACCCTAGGCGGACGGTGGCCGCCCTGA	AvrII
eryBVR	GTGGATCCGGAAACCGCTAGCCGGCGTG	BamHI
actIF	AAACAATTGCTCGAGTTTAAACAGCTCGG	MfeI
actIR	AAAGATCTCCTGCAGGTACCTAGGTTAATTAATCGATGCGTTC	BglII/SbfI/KpnI/AvrII/PacI
pikCF	AAGAATTCCTAGGTTTCCGTTCCGCTTCCGGCCCGGT	EcoRI/AvrII
pikCR	AAGAATTCGTAATGGGTGACGTGCGGGTTCAA	EcoRI

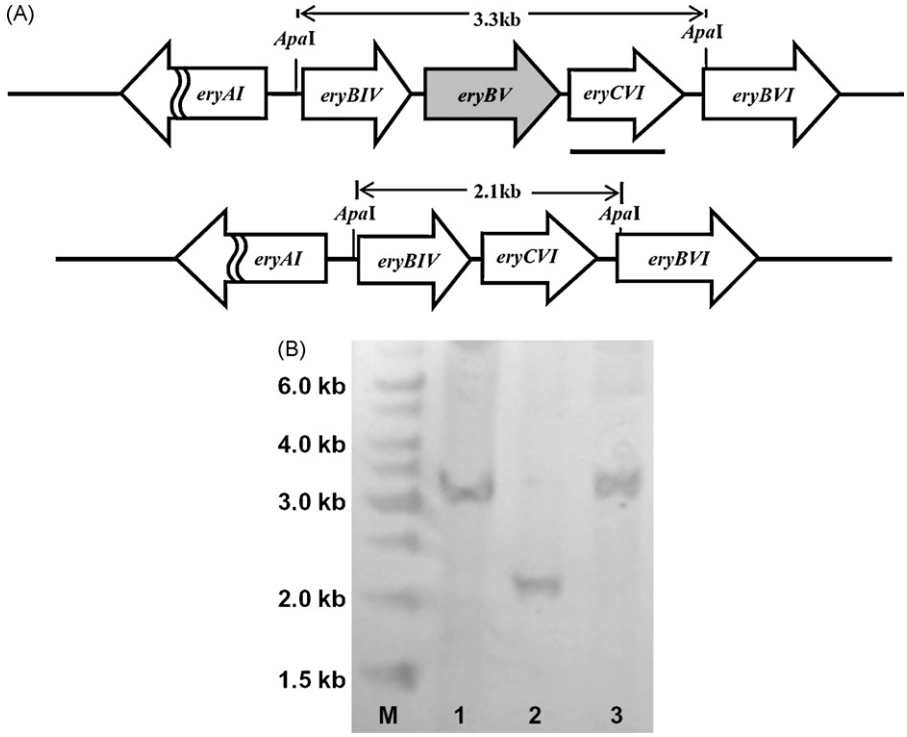

Fig. 2. (A) Restriction maps of genomic DNA from *Sac. erythraea* wild-type and *eryBV* deletion mutant YJ584. The solid line below the gene map indicated probe region. (B) Southern hybridization analysis of ApaI-digested genomic DNA from *Sac. erythraea* wild-type and the mutant using DIG-labeled DNA fragment downstream to *eryBV* as a probe. 1, Wild-type (3.3-kb); 2, *Sac. erythraea* YJ584 (2.1-kb); 3, revertant from the single crossover (3.3-kb).

complementation of *eryBV* deletion mutant was verified through restoration of erythromycin production (~80%) from the *Sac. erythraea* YJ584/pYJ591 at similar level to the wild-type (Fig. 3C), and mass spectra of ErA (**1**) and ErB (**4**) produced were also obtained (Fig. 4A and B).

Heterologous expression of PikC in *Sac. erythraea* YJ584 was expected to produce further hydroxylated form of DesEB (**2**) owing to the versatile substrate-flexibility of PikC. DesEA (**3**) production in the PikC expressing mutant, *Sac. erythraea* YJ584/pYJ586, was confirmed (Figs. 3D and 4D). Although the regio- and stereochemistry of the introduced hydroxyl group of DesEA (**3**) were not determined due to its low production (~50 $\mu g\,l^{-1}$), the modified structure of DesEB (**2**) by PikC was deduced from its intrinsic catalytic specificity (Xue et al., 1998a,b).

EryK and PikC catalyze C-12 hydroxylation of the 14-membered ring macrolides erythromycin D and narbomycin, respectively (Lambalot and Cane, 1995; Xue et al., 1998a,b), and the hydroxyl group attached to C-12 position in each macrolide represents the

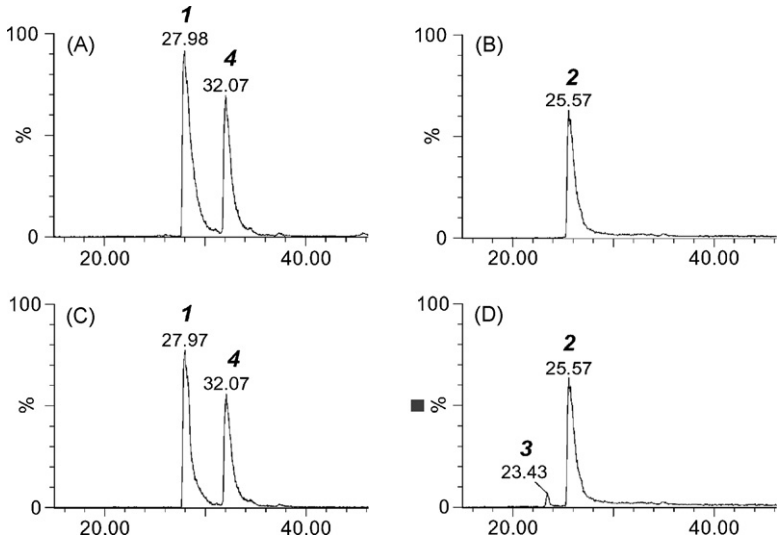

Fig. 3. HPLC-ESI-MS/MS analyses of ethyl acetate extracts from *Sac. erythraea* strains cultivated in 50 ml of TSB media at 32 °C for 5 days. A reversed-phase Nova-Pak C_{18} (4.0 μm, 150 mm × 3.9 mm, Waters) column, flow rate at 180 $\mu l\,min^{-1}$, and the gradient system using water and 80% acetonitrile with both 5 mM (w/v) ammonium acetate and 0.05% (v/v) acetic acid were employed. (A) *Sac. erythraea* wild-type; (B) *eryBV* deletion mutant YJ584; (C) *eryBV* complemented mutant YJ584/pJY591; (D) *pikC* integrated mutant YJ584/pYJ586.

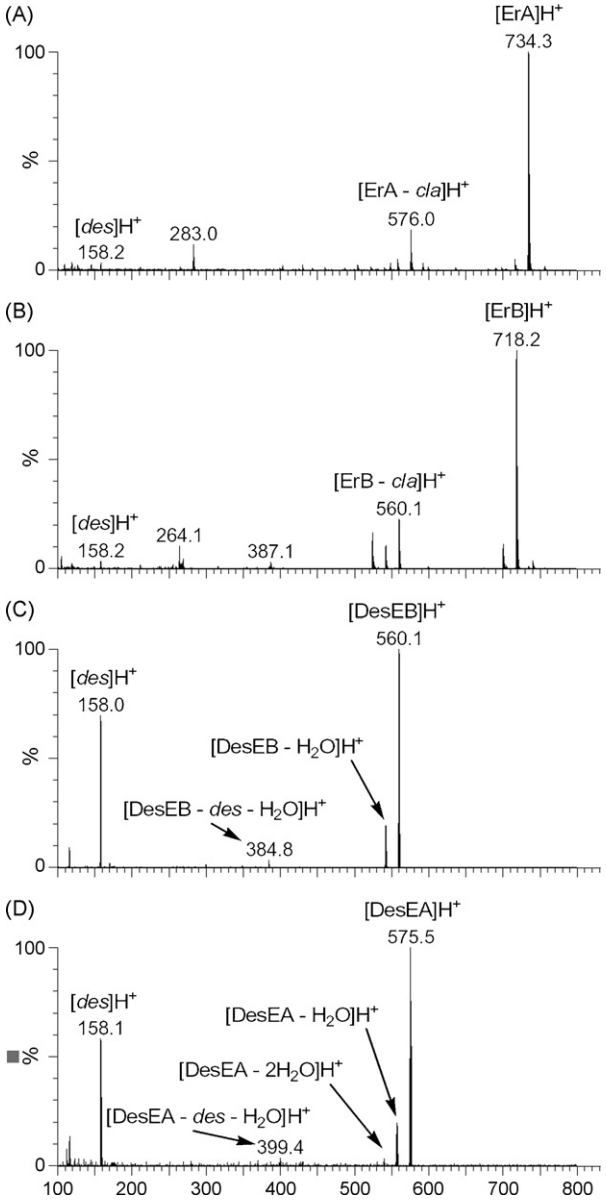

Fig. 4. Mass spectra of (A) erythromycin A (ErA): a parent ion $[ErA]H^+$ at *m/z* 734.3 and product ions $[ErA\text{-}H_2O]H^+$, [ErA-cladinose]H^+, and [desosamine]H^+ at *m/z* 716.0, 576.0, and 158.2, respectively; (B) erythromycin B (ErB): $[ErB]H^+$ at *m/z* 718.2 and products [ErB-cladinose]H^+ and [desosamine]H^+ at *m/z* 560.1 and 158.2; (C) 5-*O*-desosaminyl erythronolide B (DesEB) from the extract of *Sac. erythraea* YJ584: [DesEB]H^+ at *m/z* 560.1 and products $[DesEB\text{-}H_2O]H^+$, $[DesEB\text{-desosamine-}H_2O]H^+$, and [desosamine]$H^+$ at *m/z* 542.0, 384.8, and 158.0, respectively; (D) 5-*O*-desosaminyl erythronolide A (DesEA) from the extract of *Sac. erythraea* YJ584/pYJ586: [DesEA]H^+ at *m/z* 575.5 and products $[DesEA\text{-}H_2O]H^+$, $[DesEA\text{-}2H_2O]H^+$, $[DesEA\text{-desosamine-}H_2O]H^+$, and [desosamine]$H^+$ at *m/z* 558.0, 540.1, 399.4, and 158.1, respectively. The abbreviations *cla* and *des* represent L-cladinose and D-desosamine.

same R-configuration (Harris et al., 1965; Ogura et al., 1975). From our results, EryK seems not to hydroxylate DesEB (**2**), indicating its relatively strict substrate specificity (Lambalot and Cane, 1995). These findings are consistent with previous findings on failure of DesEA (**3**) production in *eryBV* deletion mutant (Gaisser et al., 2000), and no detection of C-12 hydroxylated product in the 3-rhamnosyl erythromycin derivatives when complemented with OleG2 in *eryBV* deletion mutant (Doumith et al., 1999). Although DesEA (**3**) was found as a minor derivative in mother liquor concentrates from commercial crystallization of erythromycin (Kibwage et al., 1987),

Table 2
List of plasmids and strains used in this study

Plasmids	Description of construction	References
pHGF7505	A cloning vector with *act*I promoter	Yu et al. (2001)
pWHM3	A shuttle vector used for gene disruption and expression	Vara et al. (1989)
pYJ584[a]	*eryBV* deletion plasmid	In this study
pYJ00[b]	*erm*E-distal part cloned to pLitmus28	In this study
pYJ585[c]	pWHM3 derivative carrying *act*I promoter and multi-cloning site	In this study
pYJ586	pYJ585 derivative for PikC expression	In this study
pYJ591	pYJ585 derivative for EryBV expression	In this study
Strains	**Description**	**References**
Sac. erythraea NRRL 2338	Wild type (red variant)	Weber et al. (1985)
Sac. erythraea YJ584	*eryBV* deletion mutant of *Sac. erythraea*	In this study
Sac. erythraea YJ584/pYJ586	Integration of pYJ586 in *Sac. erythraea* YJ584	In this study
Sac. erythraea YJ584/pYJ591	Integration of pYJ591 in *Sac. erythraea* YJ584	In this study

[a] A 1.0-kb BamHI–SpeI and a 0.9-kb NheI–XbaI PCR-amplified fragment containing the upstream and downstream region to *eryBV* were concurrently ligated into Litums28 (digested with BamHI/XbaI), respectively, following cloning of a 1.9-kb BamHI–XbaI fragment from the resulting plasmid into corresponding sites in pWHM3.

[b] A 1.5-kb PCR-amplified fragment from genomic DNA of the *Sac. erythraea* containing the *erm*E-distal part was ligated into Litmus28 (digested with EcoRI/NsiII).

[c] A 1.5 kb MfeI–BglII PCR-amplified fragment from pHGF7505 containing *actI* promoter and its cognate regulator gene *act*II-ORF4 was cloned into pWHM3 (digested with EcoRI/BamHI). New restriction sites: BglII, SbfI, KpnI, AvrII, and PacI were created by the actIR primer in pYJ585.

there has been no other report on DesEA (**3**) production from wild-type *Sac. erythraea* or mutants. The PikC with similar function to EryK but having more relaxed substrate-specificity than EryK could introduce the C-12 hydroxyl group into DesEB (**2**) to generate DesEA (**3**). The low conversion (<10%) of DesEB (**2**) to the target compound DesEA (**3**) using expression of this heterologous PikC shows need for improvement. Further study on promoting its catalytic activity by directed evolution and/or site-directed mutagenesis (Lee et al., 2006a,b) could be beneficial for enhancing the production level.

In summary, our results demonstrate an advantage of combinatorial biosynthesis which can easily provide more efficient semi-synthetic precursor to avoid the lengthy chemical synthesis. Using the substrate-flexible post-PKS tailoring enzymes such as PikC would provide a valuable tool producing unnatural polyketides which can be potentially cost-effective precursors for further chemical modifications.

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