

Designed Biosynthesis of 36-Methyl-FK506 by Polyketide Precursor Pathway Engineering

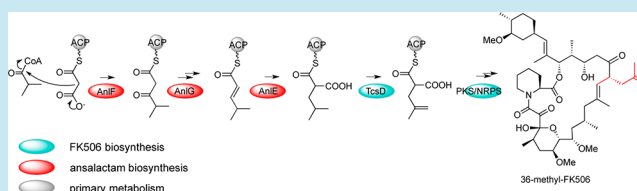
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ABSTRACT: The polyketide synthase (PKS) biosynthetic code has recently expanded to include a newly recognized group of extender unit substrates derived from α,β -unsaturated acyl-CoA molecules that deliver diverse side chain chemistry to polyketide backbones. Herein we report the identification of a three-gene operon responsible for the biosynthesis of the PKS building block isobutyrylmalonyl-CoA associated with the macrolide ansalactam A from the marine bacterium *Streptomyces* sp. CNH189. Using a synthetic biology approach, we engineered the production of unnatural 36-methyl-FK506 in *Streptomyces* sp. KCTC 11604BP by incorporating the branched extender unit into FK506 biosynthesis in place of its natural C-21 allyl side chain, which has been shown to be critical for FK506's potent immunosuppressant and neurite outgrowth activities.

KEYWORDS: biosynthesis, crotonyl-CoA carboxylase, FK506, immunosuppressant, polyketide synthase



FK506 (Tacrolimus, Prograf) is a clinically relevant drug that blocks lymphocyte activation by forming a complex with the immunophilin FKBP12 and the protein phosphatase calcineurin.¹ The bacterial natural product is assembled by the polyketide synthase (PKS) FkbA-C and nonribosomal peptide synthetase FkbP enzyme complex in *Streptomyces* sp. MA6548.² This multimodular enzymatic machinery condenses 4,5-dihydroxycyclohex-1-enecarboxylic acid, two malonyl-CoAs, two methoxymalonyl-acyl carrier proteins (-ACP), five methylmalonyl-CoAs, allylmalonyl-CoA, and a pipercolate molecule through successive rounds of condensation reactions.^{3–5} A high degree in the complexity of FK506's chemical structure is due to specific incorporation of these diverse precursors, which can be changed to alter its biological activity.⁶

Polyketide backbones have been engineered successfully in the past through building block substitutions. Precursor selectivity for starter and extender units can be predicted from modular acyltransferase (AT) domains, which perform gate-keeping functions.^{7,8} Substrate tolerance of the AT domain and promiscuity of downstream enzymes furthermore permit certain flexibility in the biosynthetic route.⁹ These features and an assorted repertoire of priming carboxylic acid substrates¹⁰ have afforded a genetic blueprint to precursor-directed combinatorial biosynthesis of rationally designed polyketide molecules.¹¹ Complex polyketides can be further modified beyond starter unit substitutions as shown in an early study by Stassi et al. in which the methylmalonate-specific AT present in the erythromycin PKS was replaced by an ethylmalonate-specific AT to generate 6-desmethyl-6-ethyl-erythromycin. Production of the ethyl-substituted analogue required supplementation with the requisite precursor in which the extender

unit substrate ethylmalonyl-CoA was generated *in vivo* after expression of a crotonyl-CoA reductase/carboxylase (CCR).¹² This example elegantly demonstrated a nonchemical complementation approach to provide an unnatural polyketide. However, the scope of polyketide engineering efforts has been restricted due to the combination of extender unit availability and AT domain specificity.¹³ Only six natural malonate derivatives with substitutions (e.g., alkyl, hydroxy, amino) at the C2 position were identified prior to 2009.¹⁴

Recently, we discovered a general pathway to a new group of extender units,¹⁵ which are accessed from α,β -unsaturated acyl-CoA substrates in a CCR-dependent manner. This paradigm shift led to an increased appreciation of polyketide extender unit diversity and opened the door for new synthetic biology approaches to generate hybrid polyketide molecules. Genes for unusual precursor biosynthesis, such as allylmalonyl-CoA (per FK506 synthesis)^{3,16} and chloroethylmalonyl-CoA (salinosporamide A),¹⁷ are associated with respective secondary metabolite gene clusters and in many cases are even organized in specialized operons. These gene cassettes are conveniently suited for *plug and play* engineering of polyketide biosynthetic pathways. Herein we report the engineered biosynthesis of 36-methyl-FK506 by replacing the endogenous allylmalonyl-CoA pathway with an exogenous pathway encoding isobutyrylmalonyl-CoA synthesis.

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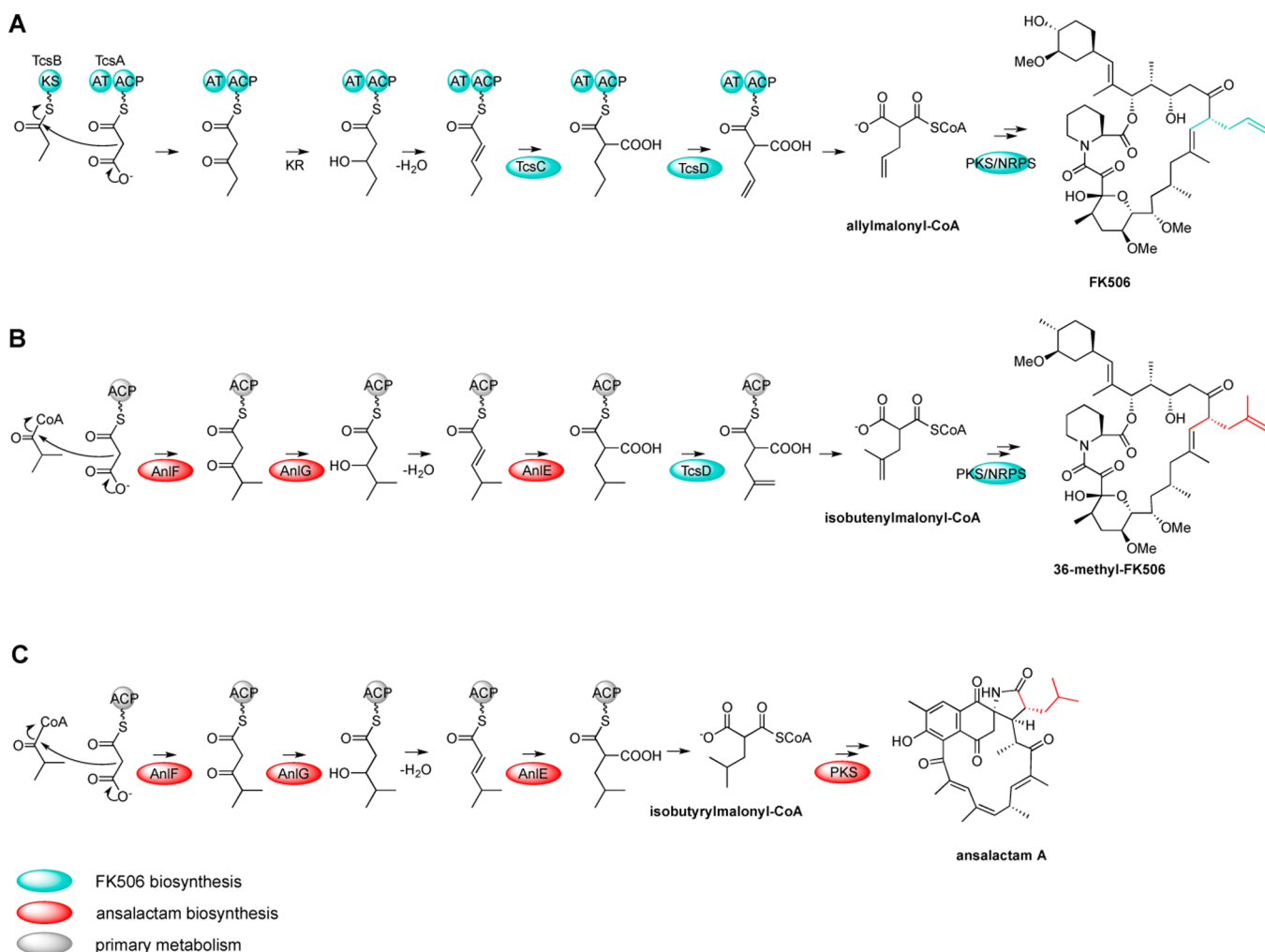


Figure 1. Genetic engineering approach toward 36-methyl-FK506 production. (a) The FK506 producer *Streptomyces* sp. KCTC 11604BP synthesizes allylmalonyl-CoA, which derives from a multistep pathway involving the ketosynthase TcsB, the cognate acyltransferase and acyl-carrier protein TcsA, the crotonyl-CoA carboxylase TcsC, and the dehydrogenase TcsD. (b) The enzymatic machineries for FK506 (panel a) and isobutyrylmalonyl-CoA (panel c) biosynthesis were combined from two different streptomycetes to generate the hybrid pathway of 36-methyl-FK506 in *Streptomyces* sp. KCTC 11604BP $\Delta tcsB$. (c) The ansalactam producer *Streptomyces* sp. CNH189 assembles isobutyrylmalonyl-CoA using the FabH-like β -ketoacyl-ACP-synthase AnlF, the dehydrogenase AnlG, and the crotonyl-CoA carboxylase AnlE.

Identification of the Isobutyrylmalonyl-CoA Biosynthetic Gene Cassette. Recently the pathway to the allyl side chain in FK506 was independently characterized by Petkovic³ and our group¹⁶ through a series of chemical, biochemical, and genetic experiments in which the dedicated ketosynthase TcsB initiates a discrete, multistep enzymatic pathway to allylmalonyl-CoA (Figure 1a). Using the *tcsB* deletion mutant strain, we produced two novel analogues, 36-fluoro-FK520 and 36-methyl-FK506, through chemical complementation with 4-fluorocrotonate and 4-methylpentanoate, respectively.¹⁶ The exogenously added precursors were converted *in vivo* to their respective α -substituted malonyl-CoAs with the help of the allylmalonyl-CoA CCR TcsC. In the case of 4-methylpentanoic acid, we hypothesize that the resultant isobutyrylmalonyl-CoA (ibMCoA) is further acted upon by the dehydrogenase TcsD to ultimately provide isobut-2-enylmalonyl-CoA for enzymatic assimilation into 36-methyl-FK506. Since the 36-methyl derivative exhibits improved neurite outgrowth activity in comparison with the parent natural product FK506, we set about to genetically engineer its construction without the requirement of chemical complementation (Figure 1b).

Polyketides with isobutyryl side chains are rare and include two recently characterized ansamycin compound families, the ansalactams¹⁸ and divergolides.¹⁹ Isotope labeling experiments showed that this biosynthetic unit is derived from isobutyrate and acetate rather than *L*-leucine. We postulate, in the context of ansalactam A biosynthesis, that the β -ketoacyl-ACP-synthase (KASIII) AnlF directly elongates isobutyryl-CoA with malonyl-ACP, which parallels the initiation reaction of fatty acid biosynthesis in streptomycetes. Subsequent AnlG-catalyzed dehydrogenation and dehydration would yield 4-methyl-2-pentenoyl-CoA, which is then reductively carboxylated by the crotonyl-CoA carboxylase homolog AnlE to afford ibMCoA (Figure 1c). To probe for the molecular mechanism of ibMCoA biosynthesis, we analyzed the draft genome of the ansalactam producer *Streptomyces* sp. CNH189, which we previously reported in the context of co-produced merochlorin antibiotics.²⁰ Bioinformatic analysis of the genome sequence identified a type I PKS gene locus containing biosynthetic genes consistent with the ansamycin typical starter unit 3,5-aminohydroxybenzoic acid.²¹ The gene cluster also included a small operon coding for the crotonyl-CoA carboxylase (CCR)

AnIE, the FabH-like β -ketoacylsynthase (KSIII) AnIF, and the dehydrogenase (DH) AnIG, which is consistent with the recently reported ibMCoA gene set associated with the divergoid macrolides.¹⁹ Phylogenetic analysis revealed that AnIE is closely related to CCRs that reductively carboxylate α,β -unsaturated acyl-CoA substrates to generate C2-substituted malonyl units.¹⁵ Therefore *anIE*, *F*, and *G* are predicted to encode enzymes for a CCR-dependent pathway to the isobutyrylmalonyl-CoA PKS substrate in ansalactam A biosynthesis.

Biosynthesis of 36-Methyl-FK506 through a Genetic Approach. To introduce the ibMCoA pathway into the FK506 producer for the engineered production of 36-methyl-FK506 (Figure 1b), we cloned the three-gene cassette *anIE–G* from the ansalactam producer strain *Streptomyces* sp. CNH189 and created constructs based on the integrative vector pSET152. Gene expression was placed under control of the native promoter in vector pMW1 and the constitutive *ermE** promoter in vector pLD6 (Figure 2). The plasmids were

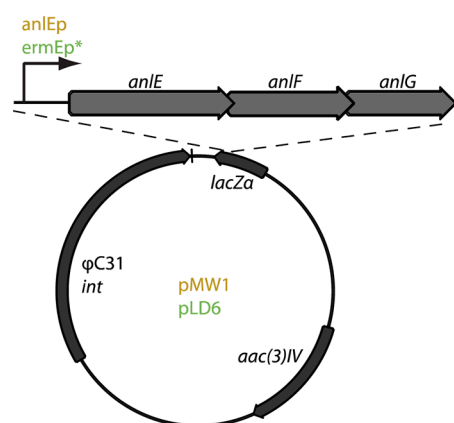


Figure 2. Expression constructs containing the ibMCoA gene cassette. The three-gene operon *anIE–G* was amplified from the ansalactam producer strain *Streptomyces* sp. CNH189 and cloned into pSET152 containing the native upstream region (orange) or the *ermE* sequence (green) affording the expression constructs pMW1 and pLD6, respectively.

conjugated into the mutant *Streptomyces* sp. KCTC 11604BP Δ *tcsB*, in which background production of FK506 was eliminated due to the deletion of the KS TcsB (Figure 3a,b). Prior to plasmid introduction into the Δ *tcsB* mutant, we observed trace levels of the target molecule, 36-methyl-FK506, at the limit of detection. This observation suggested that endogenous 3-keto-4-methylpentyl thioesters from branched chain fatty acid synthesis may be synthesized and diverted to ibMCoA through the TcsC-catalyzed reductive carboxylation of 4-methyl-2-pentenoyl-CoA.

We previously reported the mutasynthetic production of 36-methyl-FK506 upon chemical supplementation of the culture with 4-methylpentanoic acid;¹⁶ however, the *in vivo* production levels at 0.06 ± 0.009 mg/L were modest (Figure 3c). Upon chromosomal integration of vectors pLD6 and pMW1, the production of 36-methyl-FK506 was significantly increased 6-fold to 0.36 ± 0.047 mg/L and 10-fold to 0.60 ± 0.089 mg/L, respectively. (Figure 3d,e). Highest production levels were measured when the ibMCoA gene cassette remained under control of the native pathway promoter, which resulted in 2-fold higher yields compared to FK506 production in the native

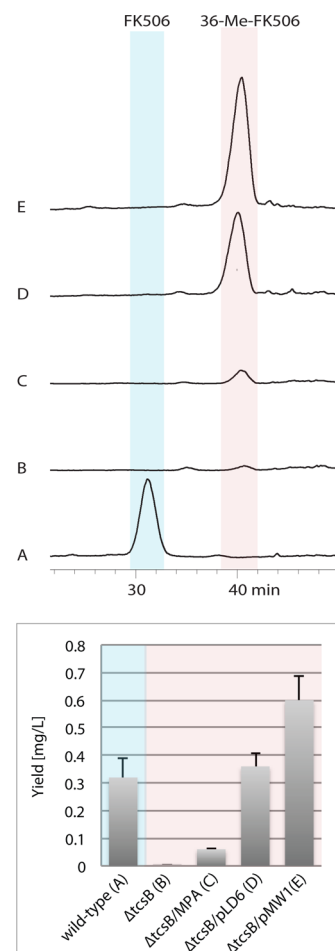


Figure 3. Production of 36-methyl-FK506 via heterologous expression of the ibMCoA pathway in a *Streptomyces* sp. KCTC 11604BP Δ *tcsB* mutant strain. Representative HPLC–ESI-MS chromatograms visualized by selected ion monitoring for FK506 (m/z 821 [$M + NH_4$]⁺; in blue) and 36-Me-FK506 (m/z 835 [$M + NH_4$]⁺; in red) in top panel. Results were obtained from five independent cultivation experiments of (A) the FK506 producing wild-type strain, (B) *Streptomyces* sp. KCTC 11604BP Δ *tcsB*, (C) the Δ *tcsB* mutant strain supplemented with 4-methylpentanoic acid (MPA), and (D) the *tcsB* deletion strain with *ermE**-p-ibMCoA biosynthetic genes (pLD6) and (E) with the native promoter-ibMCoA biosynthetic genes (pMW1). Production levels of FK50 (in blue) and 36-Me-FK506 (in red) are graphically displayed in the bottom panel.

host when similarly grown on solid media (0.32 ± 0.069 mg/L). The industrially optimized strain *Streptomyces* sp. TST10, which overproduces FK506 at approximately 900 mg/L in liquid culture,²² could provide the chassis for optimized 36-methyl-FK506 biosynthesis.

The successful engineering of 36-methyl-FK506 can be attributed to relaxed specificity of the native dehydrogenase TcsD and the AT4 domain in FkbB toward ibMCoA. The catalytic machinery of FK506 effectively accepts the unnatural building block ibMCoA in place of the native propylmalonyl-CoA, converts it to an unsaturated C4-methylated substrate, and incorporates it into the growing polyketide chain. These experiments confirm the metabolic function of the *anIE–G* operon and even demonstrate the *plug and play* character of this gene cassette in polyketide redesign.

Establishing a Tool Box for Future Engineering Efforts. Tacrolimus and structural analogues have therapeutic

potential beyond their current application as immunomodulators, e.g., as drugs in inflammatory diseases and neurological disorders, and as antimicrobial agents, which indicates that the highly functionalized FK506 pharmacophore exhibits multiple modes of action.^{23,24} To harvest their full medicinal potential, large chemistry efforts have been devoted to generating FK506 analogues,²⁵ while biosynthetic engineering efforts have been mostly limited to the structurally related immunosuppressants FK520 (ascomycin) and rapamycin.^{26,27} FK506 biosynthesis has only recently been subjected to genetic engineering efforts, owing to the successful pathway elucidation of the unusual extender unit allylmalonyl-CoA.^{3,16,28,29}

This rare natural PKS substrate belongs to the newly established CCR group of α -substituted malonates that are produced from specialized biosynthesis gene operons that accompany the cognate PKS pathway genes.¹⁵ A complementary system to a broad variety of extender units was recently established with variants of the promiscuous acyl-CoA synthetase MatB to engineer natural and non-natural PKS substrates.³⁰ This study reported that *trans*-ATs are tolerant toward non-natural extender units. To further open the door for polyketide bioengineering via the incorporation of tailored extender units, which can impact biological function as in FK506 and salinosporamide A or introduce chemoselective ligation handles, will require the tailoring of native AT domains that select the substrate for incorporation into the growing polyketide molecule.

METHODS

DNA Sequencing and Computer-Assisted Sequence Analysis. *Streptomyces* sp. CNH189 genomic DNA was sequenced using paired-end Illumina DNA sequencing as reported previously.²⁰ The software program Geneious was used for bioinformatic analysis.³¹

Accession Codes. The DNA sequences were deposited in GenBank under accession number JX867614 for the crotonyl-CoA carboxylase gene *anLE*, JX867615 for the 3-oxoacyl-(acyl-carrier-protein)-synthase gene *anIF*, and JX867616 for the 3-hydroxybutyryl-CoA dehydrogenase gene *anIG*.

Construction of Expression Vectors pLD06 and pMW1. For heterologous expression of the *ibMCoA* biosynthetic gene cassette, we employed the integrative vector pSET152. Two constructs were designed containing either the constitutively expressed *Streptomyces* promoter *ermE**p (pLD06) or the native *ibMCoA* promoter fragment (pMW1). The *ermE**p promoter was isolated by PCR from pUWL201 using primers GCATAACTAGTGCAGTGTCCGTTTCGAG (SpeI) and CTTGATCTAGAGGATCCTACCAACCGGCAC (XbaI). The fragment (191 bp) was digested and cloned into the XbaI restriction site of pSET152 to give vector pLD02. Subsequently the *ibMCoA* gene cassette (3382 bp) including the native ribosome binding site was PCR amplified from *Streptomyces* sp. CNH189 genomic DNA using primers GTCGTCTAGAAGAACCAGATCGTTTGACCAG (XbaI) and CTAGGAATTCATCAGTCGGTCAGGTCGTTTC (EcoRI). The fragment was then cloned into pLD02 to yield expression vector pLD06. For construction of vector pMW1, the same gene cassette including its native promoter region (169 bp) and ribosome binding site were amplified from genomic DNA using primers GTCGTCTAGAGGTCCTTTTCGCTCAAGACC (XbaI) and CTAGGAATTCATCAGTCGGTCAGGTCGTTTC (EcoRI). The fragment was subsequently cloned into the XbaI and EcoRI

restriction sites of pSET152. Both inserts were sequenced to confirm their integrity.

Generation of Exconjugants. The constructs pLD06 and pMW1 were transferred into *Escherichia coli* S17-1 and introduced into *Streptomyces* sp. KCTC 11604BP $\Delta tcsB$ by intergeneric conjugation.³² Apramycin resistance mutants were selected and designated as *Streptomyces* sp. KCTC 11604BP $\Delta tcsB$ /pLD06 or pMW1.

Analysis of FK506 Congeners. For production of 36-methyl FK506, the *Streptomyces* sp. KCTC 11604BP $\Delta tcsB$ strain and mutants expressing isobutyrylmalonyl-CoA biosynthetic genes were cultivated at 28 °C for 5 days on R2YE agar.¹⁶ In case of the *Streptomyces* sp. KCTC 11604BP $\Delta tcsB$ mutant, 4-methylpentanoic acid (Sigma) was added to R2YE solid medium at a final concentration of 10 mM at the time of inoculation. The grown cultures (50 mL) of the mutant strains were mixed with 1 vol of MeOH and shaken at room temperature for 12 h. The cell debris was removed by centrifugation before rotary evaporation. The MeOH extract residues were dissolved in 1 vol of water, and then extracted with 1 vol of EtOAc. The organic extract was evaporated to dryness under reduced pressure and then dissolved in 0.2 mL of MeOH for HPLC–ESI–MS/MS analysis. Samples were separated on an ACQUITY UPLC BEH C₁₈ column (50 mm \times 2.1 mm, 1.7 μ m; Waters) interfaced with a Waters/Micromass Quattro micro/MS instrument tracing by MS/MS using a gradient of MeCN at a flow rate of 0.08 mL/min over 70 min starting with 80% (v/v) aqueous MeCN containing 10 mM ammonium acetate and 0.1% acetic acid. Tracing was done by MS/MS operated in multiple reactions monitoring mode choosing mass pairs specific for the selected analytes to detect the transition from parent ion as an ammonium adduct to product ion: 835 > 590 for 36-methyl FK506.³³ Quantification of 36-methyl-FK506 was measured by comparison with authentic FK506 using HPLC–ESI–MS analysis in which the detection limit was established at 0.2 μ g/L. Five independent experiments were carried out under identical conditions.

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Kissinger, C. R.; Parge, H. E.; Knighton, D. R.; Lewis, C. T.; Pelletier, L. A.; Tempczyk, A.; Kalish, V. J.; Tucker, K. D.; Showalter, R. E.; Moomaw, E. W., et al. (1995) Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature* 378, 641–644.

- (2) Motamedi, H., and Shafiee, A. (1998) The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506. *Eur. J. Biochem.* 256, 528–534.
- (3) Goranovic, D., Kosec, G., Mrak, P., Fujs, S., Horvat, J., Kuscer, E., Kopitar, G., and Petkovic, H. (2010) Origin of the allyl group in FK506 biosynthesis. *J. Biol. Chem.* 285, 14292–14300.
- (4) Andexer, J. N., Kendrew, S. G., Nur-e-Alam, M., Lazos, O., Foster, T. A., Zimmermann, A. S., Warneck, T. D., Suthar, D., Coates, N. J., Koehn, F. E., Skotnicki, J. S., Carter, G. T., Gregory, M. A., Martin, C. J., Moss, S. J., Leadlay, P. F., and Wilkinson, B. (2011) Biosynthesis of the immunosuppressants FK506, FK520, and rapamycin involves a previously undescribed family of enzymes acting on chorismate. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4776–4781.
- (5) Wu, K., Chung, L., Revill, W. P., Katz, L., and Reeves, C. D. (2000) The FK520 gene cluster of *Streptomyces hygroscopicus* var. *ascomyceticus* (ATCC 14891) contains genes for biosynthesis of unusual polyketide extender units. *Gene* 251, 81–90.
- (6) Koehn, F. E. (2012) Biosynthetic medicinal chemistry of natural product drugs. *Med. Chem. Commun.* 3, 854–865.
- (7) Haydock, S. F., Aparicio, J. F., Molnar, I., Schwecke, T., Khaw, L. E., Konig, A., Marsden, A. F., Galloway, I. S., Staunton, J., and Leadlay, P. F. (1995) Divergent sequence motifs correlated with the substrate specificity of (methyl)malonyl-CoA:acyl carrier protein transacylase domains in modular polyketide synthases. *FEBS Lett.* 374, 246–248.
- (8) Petkovic, H., Lill, R. E., Sheridan, R. M., Wilkinson, B., McCormick, E. L., McArthur, H. A., Staunton, J., Leadlay, P. F., and Kendrew, S. G. (2003) A novel erythromycin, 6-desmethyl erythromycin D, made by substituting an acyltransferase domain of the erythromycin polyketide synthase. *J. Antibiot.* 56, 543–551.
- (9) Fischbach, M. A., and Clardy, J. (2007) One pathway, many products. *Nat. Chem. Biol.* 3, 353–355.
- (10) Moore, B. S., and Hertweck, C. (2002) Biosynthesis and attachment of novel bacterial polyketide synthase starter units. *Nat. Prod. Rep.* 19, 70–99.
- (11) Goss, R. J., Shankar, S., and Fayad, A. A. (2012) The generation of “unnatural” products: synthetic biology meets synthetic chemistry. *Nat. Prod. Rep.* 29 (8), 870–889.
- (12) Stassi, D. L., Kakavas, S. J., Reynolds, K. A., Gunawardana, G., Swanson, S., Zeidner, D., Jackson, M., Liu, H., Buko, A., and Katz, L. (1998) Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7305–7309.
- (13) Pohl, N. L., Hans, M., Lee, H. Y., Kim, Y. S., Cane, D. E., and Khosla, C. (2001) Remarkably broad substrate tolerance of malonyl-CoA synthetase, an enzyme capable of intracellular synthesis of polyketide precursors. *J. Am. Chem. Soc.* 123, 5822–5823.
- (14) Chan, Y. A., Podevels, A. M., Kevany, B. M., and Thomas, M. G. (2009) Biosynthesis of polyketide synthase extender units. *Nat. Prod. Rep.* 26, 90–114.
- (15) Wilson, M. C., and Moore, B. S. (2012) Beyond ethylmalonyl-CoA: The functional role of crotonyl-CoA carboxylase/reductase homologs in expanding polyketide diversity. *Nat. Prod. Rep.* 29, 72–86.
- (16) Mo, S., Kim, D. H., Lee, J. H., Park, J. W., Basnet, D. B., Ban, Y. H., Yoo, Y. J., Chen, S.-w., Park, S. R., Choi, E. A., Kim, E., Jin, Y.-Y., Lee, S.-K., Park, J. Y., Liu, Y., Lee, M. O., Lee, K. S., Kim, S. J., Kim, D., Park, B. C., Lee, S.-g., Kwon, H. J., Suh, J.-W., Moore, B. S., Lim, S.-K., and Yoon, Y. J. (2011) Biosynthesis of the allylmalonyl-CoA extender unit for the FK506 polyketide synthase proceeds through a dedicated polyketide synthase and facilitates the mutasynthesis of analogues. *J. Am. Chem. Soc.* 133, 976–985.
- (17) Eustáquio, A. S., McGlinchey, R. P., Liu, Y., Hazzard, C., Beer, L. L., Florova, G., Alhamadsheh, M. M., Lechner, A., Kale, A. J., Kobayashi, Y., Reynolds, K. A., and Moore, B. S. (2009) Biosynthesis of the salinosporamide A polyketide synthase substrate chloroethylmalonyl-CoA from S-adenosyl-L-methionine. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12295–12300.
- (18) Wilson, M. C., Nam, S.-J., Gulder, T. A. M., Kauffman, C. A., Jensen, P. R., Fenical, W., and Moore, B. S. (2011) Structure and biosynthesis of the marine streptomycete ansamycin ansalactam A and its distinctive branched chain polyketide extender unit. *J. Am. Chem. Soc.* 133, 1971–1977.
- (19) Xu, Z., Ding, L., and Hertweck, C. (2011) A branched extender unit shared between two orthogonal polyketide pathways in an endophyte. *Angew. Chem., Int. Ed.* 50, 4667–4670.
- (20) Kaysser, L., Bernhardt, P., Nam, S.-J., Loesgen, S., Ruby, J. G., Skewes-Cox, P., Jensen, P. R., Fenical, W., Moore, B. S., and Merochlorins, A–D (2012) cyclic meroterpenoid antibiotics biosynthesized in divergent pathways with vanadium-dependent chloroperoxidases. *J. Am. Chem. Soc.* 134, 11988–11991.
- (21) Kang, Q., Shen, Y., and Bai, L. (2012) Biosynthesis of 3,5-AHBA-derived natural products. *Nat. Prod. Rep.* 29, 243–263.
- (22) Jung, S., Moon, S., Lee, K., Park, Y. J., Yoon, S., and Yoo, Y. J. (2009) Strain development of *Streptomyces* sp. for tacrolimus production using sequential adaptation. *J. Ind. Microbiol. Biotechnol.* 36, 1467–71.
- (23) Chattopadhyaya, S., Harikishore, A., and Yoon, H. S. (2011) Role of FK506 binding proteins in neurodegenerative disorders. *Curr. Med. Chem.* 18, 5380–5397.
- (24) Monaghan, P., Fardis, M., Revill, W. P., and Bell, A. (2005) Antimalarial effects of macrolactones related to FK520 (ascomycin) are independent of the immunosuppressive properties of the compounds. *J. Infect. Dis.* 191, 1342–1349.
- (25) Maddess, M. L., Tackett, M. N., and Ley, S. V. (2008) Total synthesis studies on macrocyclic pipercolic acid natural products: FK506, the antascomicins and rapamycin. *Prog. Drug Res.* 66 (13), 15–186.
- (26) Revill, W. P., Voda, J., Reeves, C. R., Chung, L., Schirmer, A., Ashley, G., Carney, J. R., Fardis, M., Carreras, C. W., Zhou, Y., Feng, L., Tucker, E., Robinson, D., and Gold, B. G. (2002) Genetically engineered analogs of ascomycin for nerve regeneration. *J. Pharmacol. Exp. Ther.* 302, 1278–1285.
- (27) Ritacco, F. V., Graziani, E. I., Summers, M. Y., Zabriskie, T. M., Yu, K., Bernan, V. S., Carter, G. T., and Greenstein, M. (2005) Production of novel rapamycin analogs by precursor-directed biosynthesis. *Appl. Environ. Microbiol.* 71, 1971–1976.
- (28) Chen, D., Zhang, Q., Cen, P., Xu, Z., and Liu, W. (2012) Improvement of FK506 production in *Streptomyces tsukubaensis* by genetic enhancement of the supply of unusual polyketide extender units via utilization of two distinct site-specific recombination systems. *Appl. Environ. Microbiol.* 78, 5093–5103.
- (29) Kosec, G., Goranovic, D., Mrak, P., Fujs, S., Kuscer, E., Horvat, J., Kopitar, G., and Petkovic, H. (2012) Novel chemobiosynthetic approach for exclusive production of FK506. *Metab. Eng.* 14, 39–46.
- (30) Koryakina, I., McArthur, J., Randall, S., Draelos, M., Musiol, E. M., Muddiman, D. C., Weber, T., and Williams, G. J. (2012) Poly-specific trans-acyltransferase machinery revealed via engineered acyl-CoA synthetases. *ACS Chem. Biol.*, DOI: 10.1021/cb3003489.
- (31) Kears, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., and Drummond, A. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649.
- (32) Simon, R., Priefer, U., and Puhler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Nat. Biotechnol.* 1, 784–791.
- (33) Park, J. W., Mo, S. J., Park, S. R., Ban, Y. H., Yoo, Y. J., and Yoon, Y. J. (2009) Liquid chromatography-mass spectrometry characterization of FK506 biosynthetic intermediates in *Streptomyces clavuligerus* KCTC 10561BP. *Anal. Biochem.* 393, 1–7.