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Biosynthesis of the Allylmalonyl-CoA Extender Unit for the FK506 Polyketide Synthase (PKS) Proceeds Through a Dedicated PKS and Facilitates the Mutasynthesis of Novel Analogs

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Abstract

The allyl moiety of the immunosuppressive agent FK506 is structurally unique amongst polyketides and critical for its potent biological activity. Here, we detail the biosynthetic pathway to allylmalonyl-coenzyme A (CoA), from which the FK506 allyl group is derived, based on a comprehensive chemical, biochemical and genetic interrogation of three FK506 gene clusters. A discrete polyketide synthase (PKS) with noncanonical domain architecture presumably in coordination with the fatty acid synthase pathway of the host catalyzes a multi-step enzymatic reaction to allylmalonyl-CoA via trans-2-pentenyl-acyl carrier protein. Characterization of this discrete pathway facilitated the engineered biosynthesis of novel allyl group-modified FK506 analogs, namely 36-fluoro-FK520 and 36-methyl-FK506, the latter of which exhibits improved

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Accession codes. The **1** gene clusters of *Streptomyces* sp. ATCC 55098, *Streptomyces* sp. KCTC 11604BP, and *S. kanamyceticus* KCTC 9225 were deposited in GenBank under accession numbers HM116537, HM116536, and HM116538, respectively.

Supporting Information Available: Isolation and sequencing of gene clusters; deduced functions of ORFs; gene deletion experiments; phylogenetic analyses; construction of plasmids and mutants; synthesis of SNAC esters and their ^{1}H and ^{13}C NMR spectra; synthesis of *trans*-2-pentenyl-CoA and its ^{1}H NMR spectrum; ^{13}C enrichment experiment; expression, purification and analysis of enzymes; analysis of intracellular acyl-CoAs; isolation of novel analogs and their ESI-MS/MS and NMR (^{1}H, ^{13}C, COSY, HMQC, HMBC, and ^{19}F) spectra; *in silico* docking experiments; immunosuppressive and neurite outgrowth assay. This material is available free of charge via the Internet at http://pubs.acs.org.

neurite outgrowth activity. This unique feature of FK506 biosynthesis, in which a dedicated PKS provides an atypical extender unit for the main modular PKS, illuminates a new strategy for the combinatorial biosynthesis of designer macrolide scaffolds as well as FK506 analogs.

Keywords

allylmlonyl-CoA; FK506; polyketide extender unit; mutasynthesis

INTRODUCTION

FK506 (also known as Tacrolimus) (**1**), FK520 (**2**), and rapamycin (**3**) (Figure 1A) are biosynthetically related polyketides possessing antifungal and immunosuppressive activities. Since the discovery of **1** from the culture broth of the soil bacterium *Streptomyces tsukubaensis* in 1987,1 it has become a clinically important drug used to prevent rejection of transplanted organs and in the treatment of autoimmune diseases such as atopic dermatitis.2 **1** also possesses numerous promising therapeutic potentials, which include neuroprotective3 and neuroregenerative activities.4 From a biosynthetic viewpoint, **1** has a structurally unique feature compared with **2** and **3**; to the best of our knowledge, **1** is the only polyketide which carries an allyl side chain. Whereas the complete sequencing and characterization of the **2**5 and **3**6 biosynthetic gene clusters in *Streptomyces hygroscopicus* var. *ascomyceticus* ATCC14891 and *Streptomyces hygroscopicus* NRRL5491, respectively, have facilitated genetic and biochemical studies on the biosynthesis of both molecules, only the partial sequence of the **1** gene cluster has been reported until quite recently.7‚8‚9 Consequently the biosynthetic mechanism behind the introduction of the allyl functional group unique to **1** has remained unresolved.

Because the sole difference between **1** and **2** is the C21 side chain, they should be synthesized in an analogous manner by a hybrid polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) system, which is consistent with the identical organization of their known PKS/NRPS genes (Figure 1B).5‚9 The **2** PKS incorporates the shikimate-derived 4,5-dihydroxycyclohex-1-enecarboxylic acid (DHCHC, **4**) as a starter unit in a manner similar to that of **3**10 and catalyzes ten successive polyketide chain elongation cycles with two malonyl-CoA (**5**), two methoxymalonyl-acyl carrier protein (-ACP), five methylmalonyl-CoA (**6**), and one ethylmalonyl-CoA (**7**) molecule. In the case of **1**, it has been suggested that a five-carbon PKS extender unit, potentially propylmalonyl-CoA (**8**) derived from the β-oxidation of odd-chain fatty acids instead of **7**, would be incorporated into the growing polyketide chain.11 The linear polyketide chain is then condensed with pipecolate by FkbP peptide synthetase, followed by cyclization to form the macrolide ring. The ring released from the PKS complex is further modified via C9 hydoxylation by FkbD and methylation of the hydroxyl group at C31 by FkbM (Figure 1B).5‚7‚8‚9

Here we systematically investigated the biosynthetic route generating the unique C21 allyl moiety of **1**. First, comparison of the entire biosynthetic gene clusters of **1** from three different strains with that of **2** suggested that only four of the nine newly identified genes are involved in the biosynthesis of the unique PKS extender unit, allylmalonyl-CoA (**9**). Next, in-frame gene deletion, chemical complementation, and biochemical analyses revealed the detailed biosynthetic pathway for an atypical allylmalonyl PKS extender unit. Finally, novel C21 analogs of **1** were generated through a mutasynthetic approach, and their biological activities were further evaluated. The detailed understanding of the **9** biosynthetic pathway represented here allowed us to discover a new polyketide biosynthetic machinery that synthesize this atypical extender unit, generated by a dedicated PKS, which will then be incorporated into the main modular PKS, and provides a potentially valuable toolbox for the

engineered generation of not only **1** analogs with modified C21 side chains but also novel macrolide scaffolds equipped with allyl or fluoroethyl groups.

RESULTS

Analysis of the FK506 biosynthetic gene cluster

Several streptomycete strains produce **1**.12 Approximately 100 kb of the **1** biosynthetic gene clusters and their flanking regions were sequenced from three **1**-producing strains, namely *Streptomyces* sp. ATCC 55098 (MA6858), *Streptomyces. kanamyceticus* KCTC 9225, and *Streptomyces* sp. KCTC 11604BP. Analysis of these sequences revealed that fifteen genes are well maintained and identically organized in the **2** and three **1** gene clusters (Figure 2 and Table S1, Supporting Information). The PKS and NRPS genes (*fkbC, B, P* and *A*), genes for methoxymalonyl-ACP (*fkbG, H, I, J, K,* and *L*) and DHCHC (**4**) (*fkbO*) synthesis, a 31-*O*-methyltransferase gene (*fkbM*), a C9 hydroxylase gene (*fkbD*), a regulatory gene (*fkbN*), and a type II thioesterase gene (*fkbQ*) are preserved among the clusters. The **7** biosynthetic genes in the **2** cluster (*fkbE, S,* and *U*) were not found in any of the **1** clusters. A transcriptional regulator Tcs7, which belongs to the LysR-family, was located downstream of *fkbQ* only in *Streptomyces* sp. KCTC 11604BP strain.

A comparison of the three newly sequenced **1** clusters with that of **2** revealed that four contiguous genes, *tcsA*, *tcsB*, *tcsC*, and *tcsD,* are commonly found in all **1** clusters, suggesting that only these genes are involved in the biosynthesis of the unique allyl side chain of **1** (Figure 2 and Table S1, Supporting Information). Five other genes (*tcs1*, *tcs2*. *tcs3*. *tcs4*, and *tcs5*) were found only in *Streptomyces* sp. KCTC 11604BP, and in-frame gene deletion experiments confirmed that none were involved in the biosynthesis of **1** or co-occurring **2**13 (Figure S1, Supporting Information). The genes upstream of *tcsA* and downstream of *tcs7* in strain KCTC 11604BP strain do not have any obvious role in **1** biosynthesis and are not conserved in all the sequenced **1** clusters showing that the boundary of **1** cluster had been identified.

The products of *tcsA* and *tcsB* genes compose a distinct PKS system with noncanonical domain architecture. *tcsA* gene encodes an acyltransferase (AT) and an ACP domains, and *tcsB* codes for two unusual β-ketoacyl synthase (KS) domains similar to the uncharacterized PKS system of *Burkholderia* species.14 This unusual domain organization is analogous to the type II PKS priming system comprised of an initiating KS (KSIII), AT and ACP as reported in the biosynthetic gene clusters of doxorubicin, frenolicin, and R1128,15 but unique in that both AT and ACP domains are encoded by a single *tcsA* gene. Phylogenetic analysis of the AT domain of TcsA with representative sequences showed that the TcsA AT is closely related to discrete ATs specific for **5** such as FenF in mycosubtiline gene cluster, 16 LmnG in leinamycin cluster,17 and MmpIII of mupirocin cluster18(Figure S2 and Table S2, Supporting Information). Interestingly, the TcsA ACP domain phylogenetically resembles the ACP domain of fatty acid synthase (FAS) in *Escherichia coli* and is placed between type I and type II PKS associated ACP domains (Figure S3 and Table S3, Supporting Information), which reflects the unique domain architecture of TcsA and TcsB resembling both type I and type II PKSs. Analysis of the long N-terminal KS domain (448 amino acids) of TcsB shows that it contains all the predicted active site residues, and places it between type I and type II PKS KSs. The short C-terminal TcsB KS domain (320 amino acids) is phylogenetically related to the chain length factor (CLF) or KSIII of type II PKS systems, but the conserved active site residues of the typical CLF or KSIII are not preserved (Figure S4 and Table S4, Supporting Information). TcsC shares >60% identity with crotonyl-CoA (**10**) carboxylase/reductase (CCR) shown to catalyze the reductive carboxylation of an enoyl-CoA ester in the **7** pathway.11,19 TcsC is placed phylogenetically between CCR of *Streptomyces* species, which uses **10** as a substrate, and SalG of

Salinispora tropica CNB-476, which accepts chlorocrotonyl-CoA and *trans*-2-pentenyl-CoA (**11**) (Figure S5 and Table S5, Supporting Information),20·21 suggesting that TcsC has a unique substrate specificity. TcsD shows approximately 70% sequence identity with acyl-CoA dehydrogenase of *Catenulispora acidiphila* DSM 44928 and is phylogenetically related to FkbI,5 an acyl-ACP dehydrogenase involved in the biosynthesis of methoxymalonyl-ACP of the **2** gene cluster. Detailed protein sequence alignment and phylogenetic analysis suggest that TcsD is an acyl-ACP dehydrogenase since surface residues important for CoA binding in acyl-CoA dehydrogenase are replaced with hydrophobic residues as observed in FkbI (Figure S6 and Table S6, Supporting Information).22

The allylmalonyl-CoA pathway

It has been proposed that the five-carbon PKS extender unit **8** could be synthesized by reductive carboxylation of **11**, which is likely to be derived from the β-oxidation of odd-chain fatty acids.11 However, our discovery of a distinct PKS in the **1** cluster strongly implies that the five-carbon extender unit in *Streptomyces* is PKS-derived, a finding that is consistent with a previous study showing that five carbons (C20, C21, C35, C36, and C37) of **1** are derived from acetate and propionate.23 Based on the detailed sequence analysis of these four unique *tcs* genes as well as the gene deletion, chemical complementation, and biochemical experiments described later, we propose the biosynthetic route to allylmalonyl-CoA (**9**) as shown in Figure 3.

In analogy to the "chain initiation module" of the type II PKS,15 we propose that TcsB functions as a priming KS acylated by propionyl-CoA and catalyzes the condensation with malonate loaded on TcsA. Most likely, the resulting ACP-tethered β-keto-pentanoate is converted into *trans*-2-pentenyl-ACP before the chain is further processed. Because no genes encoding β-keto processing enzymes, namely the ketoreductase and dehydratase, responsible for this reductive process were found in any of the sequenced **1** clusters, we hypothesize that these activities are shared with the FAS-like enzyme of the host, as is the case with type II PKS initiation modules.15 Indeed, SCO1815, a FabG (the β-ketoacyl-ACP reductase of fatty acid biosynthesis) homolog from the genome of *Streptomyces coelicolor* A3(2), was shown to function as the β-ketoacy l-ACP reductase component of the R1228 initiation module.24 The next reductive carboxylation reaction, giving propylmalonyl-ACP, is catalyzed by TcsC in a manner analogous to SalG.20·21 We propose that TcsD catalyzes the reaction to allylmalonyl-ACP, which is subsequently loaded onto module 4 of **1** PKS through **9**. Alternatively, TcsD might convert *trans*-2-pentenyl-ACP to (*2E*)-2,4-pentadienyl-ACP, which in turn undergoes reductive carboxylation by TcsC to allylmalonyl-ACP. Skipping the TcsD-catalyzed dehydrogenation reaction would produce **8**. No gene encoding an ACP:CoA transacylase-like enzyme, which might be required for the conversion of propylmalonyl- and allylmalonyl-ACP to **8** and **9**, respectively, was located in the **1** gene clusters.

Chemical complementation of *tcsA, tcsB, tcsC*, and *tcsD* deletion mutants

To obtain experimental evidence for the functions of *tcsA*, *tcsB*, *tcsC*, and *tcsD* in **1** biosynthesis, each postulated gene in *Streptomyces* sp. KCTC 11604BP was inactivated by in-frame deletion to avoid any polar effect (see Supporting Information for the gene construction and Tables S7 and S8 in Supporting Information). Synthetic acyl-*N*-acetylcysteamine thioesters (SNACs) that mimic the corresponding intermediates proposed in Figure 3 were next fed to each deletion mutant to probe their effect on **1** biosynthesis (Figure 4; see Supporting Information for chemical synthesis of SNAC esters and Figure S7 to S16 in Supporting Information for their ^{1}H and ^{13}C NMR spectra).

The deletion of *tcsB* led to the selective loss of **1** production in contrast to **2**, confirming the dedicated involvement of TcsB in the biosynthesis of **1**. We also observed that the production of FK523 (35-desmethyl-FK520, **12**), which can be detected only in trace amounts in the wild-type strain by HPLC–ESI–MS/MS analysis,13 was significantly increased as a result of the misincorporation of **6** in the absence of the five-carbon extender unit. Supplementing two synthetic acyl-SNACs, 3-oxopentanoyl-SNAC (**13**) and *trans*-2-pentenyl-SNAC (**14**), restored **1** production in the *tcsB* deletion mutant (ΔtcsB strain), probably after loading onto TcsA per the proposed pathway (Figure 3). **1** production was also restored by pentanoyl-SNAC (**15**), possibly via β-oxidation to **14** by acyl-CoA dehydrogenase. Lastly, restoration of **1** biosynthesis by allylmalonyl-SNAC (**16**) suggests that the *in vivo* extender unit is CoA-linked instead of ACP-linked. Although *tcsD*, which is responsible for the formation of the C36–C37 double bond of **1** as described below, is intact in the ΔtcsB strain, propylmalonyl-SNAC (**17**) supported the biosynthesis of only 36,37-dihydro-FK506 (**18**) as determined by HPLC–ESI–MS/MS.13 This result suggests that the exogenously fed carboxylated SNAC thioester **17** is not loaded onto the TcsA ACP domain, which is required for the desaturation activity of TcsD as predicted by its sequence (Figure 4A). To confirm the incorporation of an intact five-carbon extender unit into the **1** polyketide chain, [1-^{13}C]pentanoic acid (**19**) was provided as a precursor to the ΔtcsB mutant. As anticipated, ^{13}C NMR analysis of **19**-enriched **1** revealed the specific isotopic labeling of C20 at approximately 23% enrichment (Figure S17, Supporting Information). C8 and C22, corresponding to the positions where the acetate was incorporated,23 were also labeled at a lower percentage of □8–15% presumably from the degradation of **19** by β-oxidation. These acetate-derived carbons were also enriched in **2** and **12** purified from the same **19**-fed ΔtcsB mutant.

The *tcsA* deletion mutant (ΔtcsA strain) also produced only **2** and **12**. Because the 3-oxopentanoate moiety bound to ACP is believed to be processed to *trans*-2-pentenyl-ACP by the recruited FAS-like system (Figure 3), **13** did not restore **1** production in the absence of TcsA as it did in the absence of TscB (Figure 4A). Supplementation of the ΔtcsA strain with **14** and **15** resulted in the production of trace amounts of **18**, but no **1** production, thereby supporting our previous observation that the dehydrogenase TcsD operates with the ACP bound substrate. Furthermore, this finding suggests that the reductive carboxylase TcsC also prefers ACP-linked substrates but can default operate with acyl-CoAs, which is typical of enzymes in this family. As expected, the addition of **16** and **17** produced **1** and **18**, respectively (Figure 4B).

To further explore the *in vivo* function of *tcsC*, this gene was inactivated which also resulted in the selective loss of **1**. Chemical complementation of the *tcsC* deletion mutant (ΔtcsC strain) with **13**, **14**, and **15** did not restore production of **1** or **18**, thereby confirming its central role in functionalizing the five-carbon extender unit by reductive carboxylation. Again, addition of the malonates **16** and **17** yielded **1** and **18**, respectively (Figure 4C). The lack of **1** production in the ΔtcsC strain fed with **17** provides further support for the previous speculation that the TcsA ACP domain is not acylated with **17** and that TcsD is an acyl-ACP dehydrogenase.

Lastly, the *tcsD* deletion mutant (ΔtcsD strain) produced **2** and large amounts of **18**, thus confirming its central role in the formation of the C36/C37 olefin of **1**. While exogenous **13**, **14**, **15**, and **17** did not change the production profile of the ΔtcsD strain, the addition of **16** restored **1** production (Figure 4D). **18** produced in both the ΔtcsB and ΔtcsC mutants by chemical complementation was not further transformed into **1** despite the presence of *tcsA* and *tcsD*, indicating that the double bond of the C21 allyl group of **1** is not generated through post-PKS modification.

Biochemical analysis: *in vitro* reconstitution of allylmalonyl-ACP biosynthesis

To demonstrate the precise roles of TcsC and TcsD, a biochemical and mass spectral approach was employed to reconstitute and measure allylmalonyl-ACP formation *in vitro*. The recombinant TcsC and ACP domain of TcsA (ACP_{tcsA}) were expressed in *E. coli* as histidine-tagged proteins and purified by nickel-affinity chromatography. Domain boundaries of ACP_{tcsA} were chosen according to literature precedent.25 Soluble recombinant histidine-tagged TcsD was instead obtained by expression in *Streptomyces lividans* TK24. On the basis of the *in vivo* mutagenesis results, the most likely substrate for TcsC or TcsD is *trans*-2-pentenyl-ACP_{tcsA}. By using the broad specificity phosphopantetheinyl transferase Sfp26 from *Bacillus subtilis* and chemically synthesized **11** (see Supporting Information for chemical synthesis and Figure S18 for its ^{1}H NMR spectrum in Supporting Information), we biochemically converted apo-ACP_{tcsA} to *trans*-2-pentenyl-ACP_{tcsA} (Figure 5A and 5B). Upon incubation with TcsC, we measured by ESI-MS its NADPH-dependent reductive carboxylation to propylmalonyl-ACP_{tcsA} (Figure 5C). A further FAD-dependent dehydrogenation reaction catalyzed by TcsD resulted in a 2-Da decrease in mass consistent with the formation of allylmalonyl-ACP_{tcsA}, a conclusion also supported by the observed shift in HPLC elution time (Figure 5D). Alternatively, incubation of *trans*-2-pentenyl-ACP_{tcsA} with TcsD generated a 2-Da lower mass consistent with the formation of (*2E*)-2,4-pentadienyl-ACP_{tcsA} at a low rate of conversion (Figure 5E), which precluded it from being reacted with TcsC due to its low yield. These results suggest that the major biosynthetic pathway leading to allylmalonyl-ACP and thus **9** is through propylmalonyl-ACP (Figure 3). Although the CoA thioesters **10** and **11** were also converted by TcsC to **7** and **8**, respectively (Figure S19, Supporting Information), the relatively low activity of TcsC toward **10** and **11** compared with *trans*-2-pentenyl-ACP_{tcsA} supports the deduced preference of TcsC for the ACP-linked substrate, although the absolute kinetic parameters for TcsC toward **10**, **11**, and *trans*-2-pentenyl-ACP_{tcsA} were not measured. The behavior of TcsC demonstrates an unprecedented example of the preference of a CCR-like carboxylase/reductase for ACP versus CoA-bound substrates. This preference for ACP-based substrates was also observed with TcsD, which did not accept **11** as an alternative substrate (Figure S19, Supporting Information).

Chemical complementation of an FK520-producing strain

FK506 (**1**) producers biosynthesize trace amounts of **12** and **18** along with **1** and **2**(Figure 1B and 6).13 Based on the in-frame deletion of the *fkbA* PKS gene that resulted in the complete loss of production of **1**-related compounds (Figure 6), a single PKS is responsible for the biosynthesis of all **1** congeners in which the AT domain of module 4 (AT4) has broad substrate specificity toward **6**, **7**, **8**, and **9**. From the high sequence homology between the AT4 domains of distinct **1** and **2** PKSs, we reasoned that exogenously added allylmalonyl-SNAC (**16**) and propylmalonyl-SNAC (**17**) could be incorporated into the **2** PKS of *S. hygroscopicus* var. *ascomyceticus* ATCC14891 to generate **1** and **18**, respectively. Indeed, a **2** producer supplemented with **16** and **17** produced **1** and **18**, respectively, confirming that **9** and **8** are the novel PKS extender units introducing allyl and propyl side chains at C21 of **1** and **18**, respectively (Figure 6). Analysis of the *in vivo* pool of **9** in the **1**-producing and nonproducing strains suggests that the concentration of intracellular **9** directs the biosynthesis of **1**. In *Streptomyces* sp. KCTC 11604BP, approximately 450 pmol/g cell wet weight of intracellular **9** was detected, whereas none was detected in *S. hygroscopicus* var. *ascomyceticus* ATCC14891 (Figure S20, Supporting Information; see Figure S21 in Supporting Information for ^{1}H NMR spectrum of the synthetic CoA ester **9**).

Mutasynthesis of FK506 analogs

The tolerance of AT4 of **1** PKS for various extender units suggested the possibility for the mutasynthesis or precursor-directed biosynthesis of novel **1** analogs containing diverse side

chains at the C21 position in place of the allyl group. Since the biosynthesis of **1** analogs is more efficient in the absence of competition from the natural extender unit, a series of carboxylic acids, including 4-halocrotonic acids, branched/4-halobutanoic acids, branched/unsaturated/5-halopentanoic acids, branched/unsaturated hexanoic acids, and heptanoic acid, were fed to the ΔtcsB strain. New metabolites were produced by feeding *trans*-2-hexenoic acid (**20**), 4-methylpentanoic acid (**21**), and 4-fluorocrotonic acid (**22**) resulting in 36,37-dihydro-37-methyl-FK506 (**23**), 36-methyl-FK506 (**24**), and 36-fluoro-FK520 (**25**), respectively (Figure 7). Among these products, **23** had been previously synthesized27 and confirmed here by ESI-MS/MS (Figure S22, Supporting Information). The novel analogs **24** and **25** were elucidated by NMR (Figures S23 to S35, Supporting Information; Tables S9 and S10, Supporting Information).

In silico docking experiments were conducted to probe the binding of the FKBP12-**24/25** complexes to calcineurin in comparison to **1** and **2**. The binding free energy of the FKBP12-**24**-calcineurin complex (−7.78 Kcal/mol) is smaller than that of FKBP12-**1**-calcineurin (−6.42 Kcal/mol), suggesting that the relatively stronger interaction of the FKBP12-**24** complex with calcineurin may lead to a higher immunosuppressive activity.28 In contrast, the binding free energy between FKBP12-**25** and calcineurin was relatively higher at −5.82 Kcal/mol (Figure S36 and Table S11, Supporting Information; see Supporting Information for detailed methods). Evaluation of interleukin-2 (IL-2) secretion from activated human T lymphocytes treated with **1**, **2**, **24**, and **25** showed that the *in vitro* immunosuppressive activity of **24** was not improved against **1** and **2** (Figure 8). On the other hand, **24** had a ~20% greater effect on neurite outgrowth in cultures of the human neuroblastoma cell line SH-SY5Y treated with nerve growth factor compared with **1** (Figure 8; Figure S37, Supporting Information).

DISCUSSION

Despite the structural novelty and biological significance of the allyl group of **1**, its detailed biosynthesis had remained unresolved until this study. Recently the "*all* subcluster" in the **1**-producing *S. tsukubaensis* NRRL 18488 was reported during the preparation of this manuscript and shown to harbor nine genes identical to *tcs* genes(Table S1, Supporting Information).29 The authors deleted the *tcsB* homolog *allK* to abolish **1** production and restored its production with synthetic allylmalonyl-SNAC (**16**), findings consistent with our results indicating the involvement of the *all* cluster in the biosynthesis of the novel polyketide building block **9**. However, the functional assignment of the other gene products involved in the carboxylation and desaturation reactions was incomplete, and the proposed biosynthetic pathway was partially incorrect. In this study we report a comprehensive *in vivo* and *in vitro* interrogation of **9** biosynthesis that represents an unprecedented strategy in nature in which a dedicated PKS provides the main modular PKS with an atypical extender unit.

The PKS system composed of TcsA and TcsB that is responsible for the biosynthesis of the five-carbon PKS extender unit exhibits hitherto unknown PKS domain architecture. Although the detailed functions of both KS domains contained in TcsB are not presently known, it is plausible that the N-terminal KS domain, in which all the predicted active sites are preserved, catalyzes the decarboxylative Claisen condensation reaction and that the C-terminal CLF-like KS domain controls the number of Claisen condensations during polyketide assembly. Also unusual is TcsC that prefers ACP-bound substrates over CoA-esters, a behavior not previously observed amongst CCR-like enzymes. Its extended substrate promiscuity toward unnatural 2-alkenyl-ACPs was fortuitous as it allowed for the successful mutasynthesis of the novel **1** analogs **24** and **25**. Whether the lack of incorporation of 4-chloro- and 4-bromocrotonic acids into **1** analogs was due to a limited

flexibility of TcsC or the AT4 domain in **1** PKS remains to be seen. The acyl-ACP dehydrogenase TcsD responsible for the generation of the terminal olefin of **9** also seems to have a certain degree of substrate tolerance based on the mutasynthesis of unnatural **24** from the saturated, branched-chain precursor **21**. Biochemical analysis shown in Figure 3 suggests that the dehydrogenation catalyzed by TcsD follows, rather than precedes, the reductive carboxylation by TcsC, although 4,5-desaturation is much less favorable than 2,3-desaturaion. However, it is at present unclear how it is plausible for a FAD-dependent TcsD catalyzes the desaturation of a relatively unactivated terminal methylene.

In addition to expanding the pool of PKS building blocks, the availability of AT domains to recognize and incorporate diverse extender units is a key challenge in the exploitation of type I PKS modularity for the diversification of polyketide structures. The incorporation of a range of extender units by AT4 of **1** PKS led us to conduct a detailed phylogenetic analysis. The allylmalonyl-specific AT4 of the **1** PKS is closely related to methylmalonyl- and ethylmalonyl-specific ATs. However, the distinctive signature motifs found by sequence alignment support the notion that **1** AT4 accepts an atypical extender unit in addition to a range of known extender units (Figure S38, Supporting Information).

The recent discovery of the new PKS extender units hydroxymalonyl- and aminomalonyl-ACP,30 haloethylmalonyl-CoAs,20 and **8**21 and the elucidation of their biosynthetic pathways has significantly expanded the pool of PKS building blocks to be used in the assembly of new polyketide scaffolds. The addition of a novel allylmalonyl PKS extender unit to the repertoire of PKS building blocks provides added potential for increasing the structural diversity of polyketides with a chemically tractable olefinic handle. The production of **1** from a **2**-producing strain supplemented with **16** demonstrates the feasibility of biologically installing an allyl group onto other polyketide scaffolds.

Practically we were able to prepare **1** analogs modified at C21, a position known to be a critical for its biological activity. Even slight modification of the C21 allyl group of **1** can significantly alter its activity. For instance, inhibition of T cell activation is the result of the binding of the FKBP12-**1** binary complex to calcineurin, an event that relies on major interactions involving C15-C17 and the C21 allyl group of **1** extending into the hydrophobic cleft located at the interface of the calcineurin A and B subunits.28 Although synthetic modifications of the C21 allyl group in **1** have provided structural derivatives,31 the mutasynthetic strategy enabled us to generate novel **1** analogs not readily assessable via semisynthesis. In particular, the fluorinated **25** is novel amongst FK506 analogs to harbor a side chain fluoro substituent since fluorine often exerts a favorable effect on the pharmacological and pharmacokinetic properties of the lead compounds.32 Our mutasynthetic strategy provides a new general strategy for the controlled fluorination of macrolide side chains that may lead to improved biological properties.

Although the immunosuppressive activity of the new branched-chain analog **24** was not improved as predicted by molecular modeling, it exhibited improved *in vitro* neurite outgrowth activity in comparison to **1**. We thus are exploring the potential of this novel neuroregenerative agent as a new molecular probe to investigate the detailed mechanism underlying neuroregeneration by neuroimmunophilin ligands. Significantly, our procedure exemplifies a new combinatorial biosynthetic method to modify structurally complex molecules such as **1** as an alternative method to chemical synthesis and to create improved biological agents.

EXPERIMENTAL SECTION

General

Bacterial strains, identification and sequencing of the FK506 (**1**) biosynthetic gene clusters, culture conditions, and materials are described in Supporting Information.

Chemicals

The chemical synthesis and structure of allylmalonyl-CoA (**9**), *trans*-2-pentenyl-CoA (**11**), 3-oxopentanoyl-SNAC (**13**), *trans*-2-pentenyl-SNAC (**14**), pentanoyl-SNAC (**15**), allylmalonyl-SNAC (**16**), propylmalonyl-SNAC (**17**), and 4-fluorocrotonic acid (**22**) are described in Supporting Information.

Construction of plasmids and mutants

tcs genes were inactivated in the **1**-producing strain *Streptomyces* sp. KCTC 11604BP by in-frame deletion via double cross-over homologous recombination. Details regarding DNA isolation and manipulation, and construction of plasmids for gene deletion and heterologous expression as well as the resulting mutant strains are described in Supporting Information (see also Tables S7 and S8).

Analysis of FK506 congeners and their analogs

1-related biosynthetic intermediates and their analogs, which were generated by **1**-producing *Streptomyces* sp. KCTC 11604BP, its deletion mutants, and deletion mutants supplemented with the SNAC thioesters **13**, **14**, **15**, **16**, and **17**, and a series of carboxylic acids, as well as **2**-producing *S. hygroscopicus* var. *ascomyceticus* ATCC 14891, were extracted with EtOAc from the fermentation broth (see Supporting Information), then analyzed by HPLC-ESI-MS/MS as described.13 Samples were separated on an ACQUITY UPLC BEH C_{18} column (50 × 2.1 mm, 1.7 μm; Waters) interfaced with a Waters/Micromass Quattro *micro*/MS instrument tracing by MS/MS using a gradient of MeCN at a flow rate of 0.2 ml/min over 50 min starting with 40% (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: 821 > 576 for **1**; 809 > 564 for **2**; 795 > 550 for **12**; 823 > 578 for **18**; 837 > 592 for **23**; 835 > 590 for **24**; and 827 > 582 for **25**. Three separate cultivations and independent extractions were performed.

Isolation and structural identification of FK506 analogs

Details regarding the isolation and characterization of new **1** analogs obtained by supplementing the *tcsB* deletion mutant with three different carboxylic acids (**20**, **21**, and **22**) are described in Supporting Information.

Mass spectrometric analysis of *in vitro* conversion of 2-pentenyl-ACP_{tcsA} into propylmalonyl-, (*2E*)-2,4-pentadienyl-, and allylmalonyl-ACP_{tcsA}

Details regarding the preparation and purification of recombinant ACP_{tcsA}, TcsC, TcsD and Sfp, as well as *in vitro* TcsC- and TcsD-mediated production and analysis of propylmalonyl-, (*2E*)-2,4-pentadienyl-, and allylmalonyl-ACP_{tcsA} from *trans*-2-pentenyl-ACP tcsA, are described in Supporting Information.

Other methods

Phylogenetic analysis and sequence alignment of the four *tcs* genes, *tcsA*, *tcsB*, *tcsC*, and *tcsD*, which are responsible for **9** biosynthesis and the AT4 domain in **1** PKS in the strain

KCTC 11604BP, are available in Supporting Information. A feeding experiment with [1-^{13}C]pentanoic acid (**19**) was conducted using the *tcsB* deletion mutant (see Supporting Information). Intracellular CoAs derived from both **1**- and **2**-producing strains were analyzed by HPLC-ESI-MS/MS as described (see Supporting Information).33 Details regarding 3-dimensional modeling, docking, and molecular dynamics simulation are described in Supporting Information. The evaluation of immunosuppressive and neurite outgrowth activities of **1** and its analogs are also described in Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

(1). Kino T, Hatanaka H, Miyata S, Inamura N, Nishiyama M, Yajima T, Goto T, Okuhara M, Kohsaka M, Aoki H, Ochiai T. J. Antibiot. (Tokyo). 1987; 40:1256–1265. [PubMed: 2445722]

(2). Parsons WH, Sigal NH, Wyvratt MJ. Ann. N. Y. Acad. Sci. 1993; 685:22–36. [PubMed: 7689805]

(3). Sierra-Paredes G, Sierra-Marcuño G. CNS Neurosci. Ther. 2008; 14:36–46. [PubMed: 18482098]

(4). Gold BG. Expert. Opin. Investig. Drugs. 2000; 9:2331–2342.

(5). Wu K, Chung L, Revill WP, Katz L, Reeves CD. Gene. 2000; 251:81–90. [PubMed: 10863099]

(6). Schwecke T, Aparicio JF, Molnár I, König A, Khaw LE, Haydock SF, Oliynyk M, Caffrey P, Cortés J, Lester JB, Böhm GA, Staunton J, Leadlay P. Proc. Natl. Acad. Sci. USA. 1995; 92:7839–7843. [PubMed: 7644502]

(7). Motamedi H, Shafiee A, Cai SJ, Streicher SL, Arison BH, Miller RR. J. Bacteriol. 1996; 178:5243–5248. [PubMed: 8752344]

(8). Motamedi H, Cai SJ, Shafiee A, Elliston KO. Eur. J. Biochem. 1997; 244:74–80. [PubMed: 9063448]

(9). Motamedi H, Shafiee A. Eur. J. Biochem. 1998; 256:528–534. [PubMed: 9780228]

(10). Lowden PA, Wilkinson B, Böhm GA, Handa S, Floss HG, Leadlay PF, Staunton J. Angew. Chem. Int. Ed. 2001; 40:777–779.

(11). Chan YA, Podevels AM, Kevany BM, Thomas MG. Nat. Prod. Rep. 2009; 26:90–114. [PubMed: 19374124]

(12). Muramatsu H, Mokhtar SI, Katsuoka M, Ezaki M. Actinomycetologica. 2005; 19:33–39.

(13). Park JW, Mo SJ, Park SR, Ban YH, Yoo YJ, Yoon YJ. Anal. Biochem. 2009; 393:1–7. [PubMed: 19539594]

(14). Holden MT, et al. Proc. Natl. Acad. Sci. USA. 2004; 101:14240–14245. [PubMed: 15377794]

(15). Hertweck C, Luzhetskyy A, Rebets Y, Bechthold A. Nat. Prod. Rep. 2007; 24:162–190. [PubMed: 17268612]

(16). Duitman EH, Hamoen LW, Rembold M, Venema G, Seitz H, Saenger W, Bernhard F, Reinhardt R, Schmidt M, Ullrich C, Stein T, Leenders F, Vater J. Proc. Natl. Acad. Sci. USA. 1999; 96:13294–13299. [PubMed: 10557314]

(17). Cheng YQ, Tang GL, Shen B. Proc. Natl. Acad. Sci. USA. 2003; 100:3149–3154. [PubMed: 12598647]

(18). El-Sayed AK, Hothersall J, Cooper SM, Stephens E, Simpson TJ, Thomas CM. Chem. Biol. 2003; 10:419–430. [PubMed: 12770824]

(19). Erb TJ, Berg IA, Brecht V, Müller M, Fuchs G, Alber BE. Proc. Natl. Acad. Sci. USA. 2007; 104:10631–10636. [PubMed: 17548827]

(20). Eustáquio AS, McGlinchey RP, Liu Y, Hazzard C, Beer LL, Florova G, Alhamadsheh MM, Lechner A, Kale AJ, Kobayashi Y, Reynolds KA, Moore BS. Proc. Natl. Acad. Sci. USA. 2009; 106:12295–12300. [PubMed: 19590008]

(21). Liu Y, Hazzard C, Eustáquio AS, Reynolds KA, Moore BS. J. Am. Chem. Soc. 2009; 131:10376–10377. [PubMed: 19601645]

(22). Watanabe K, Khosla C, Stroud RM, Tsai SC. J. Mol. Biol. 2003; 334:435–444. [PubMed: 14623185]

(23). Byrne, KM.; Shatiee, A.; Nielsen, A.; Arison, JB.; Monaghan, RL.; Kaplan, L. The biosynthesis and enzymology of an immunosuppressant, immunomycin, produced by Streptomyces hygroscopicus var. ascomyceticus. In: Nash, C.; Hunter-Cevera, J.; Cooper, R.; Eveleigh, DE.; Hamill, R., editors. Developments in industrial microbiology series. Vol. 32. Wm. C. Brown; Iowa: 1993. p. 29-45.

(24). Tang Y, Lee HY, Tang Y, Kim CY, Mathews I, Khosla C. Biochemistry. 2006; 45:14085–14093. [PubMed: 17115703]

(25). Wu N, Cane DE, Khosla C. Biochemistry. 2002; 41:5056–5066. [PubMed: 11939803]

(26). Quadri LE, Weinreb PH, Lei M, Nakano MM, Zuber P, Walsh CT. Biochemistry. 1998; 37:1585–1595. [PubMed: 9484229]

(27). Organ HM, Holmesa MA, Pisanoa JM, Starucha MJ, Wyvratta MJ, Dumonta FJ, Sinclaira PJ. Bioorg. Med. Chem. Lett. 1993; 3:657–662.

(28). Griffith JP, Kim JL, Kim EE, Sintchak MD, Thomson JA, Fitzgibbon MJ, Fleming MA, Caron PR, Hsiao K, Navia MA. Cell. 1995; 82:507–522. [PubMed: 7543369]

(29). Goranovic D, Kosec G, Mrak P, Fujs S, Horvat J, Kuscer E, Kopitar G, Petkovic H. J. Biol. Chem. 2010; 285:14292–14300. [PubMed: 20194504]

(30). Chan YA, Boyne MT 2nd. Podevels AM, Klimowicz AK, Handelsman J, Kelleher NL, Thomas MG. Proc. Natl. Acad. Sci. USA. 2006; 103:14349–14354. [PubMed: 16983083]

(31). Caufield CE. Curr. Pharm. Design. 1995; 1:145–160.

(32). Eustáquio AS, Moore BS. Angew. Chem. Int. Ed. 2008; 47:3936–3938.

(33). Park JW, Jung WS, Park SR, Park BC, Yoon YJ. J. Mass Spectrom. 2007; 42:1136–1147. [PubMed: 17565713]

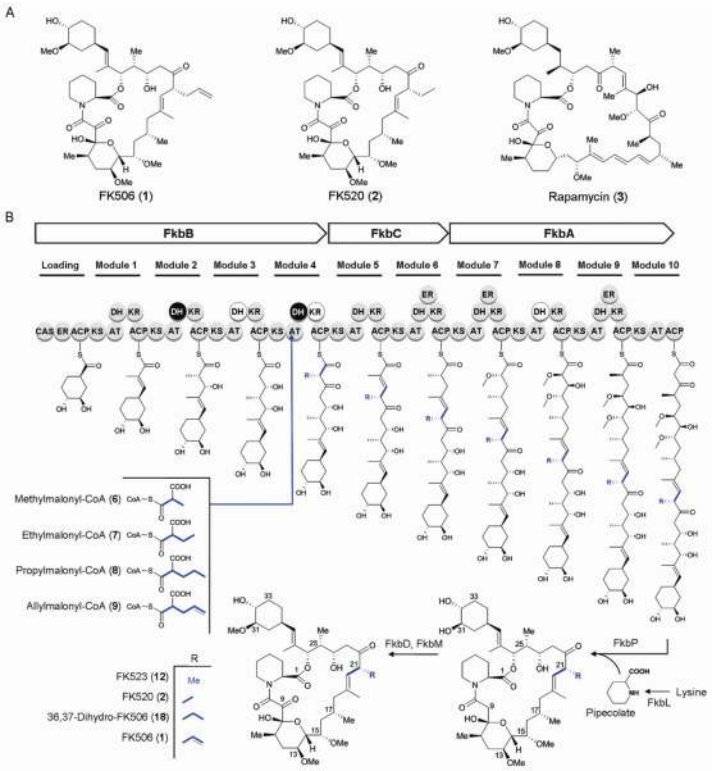

Figure 1. Structures of FK506. FK520, and rapamycin (A), and schematic representation of the FK506 PKS and biosynthesis of FK506 and its derivatives (B)

Domains within each module are represented by circles. Black and white circles indicate domains that are not predicted to be active from the final structure and domains that are nonfunctional due to deletions in the active sites, respectively. CAS, CoA synthetase; KS, ketoacyl synthase; AT, acyl transferase; DH, dehydratase; ER, enoyl reductase; KR, keto reductase; ACP, acyl carrier protein.

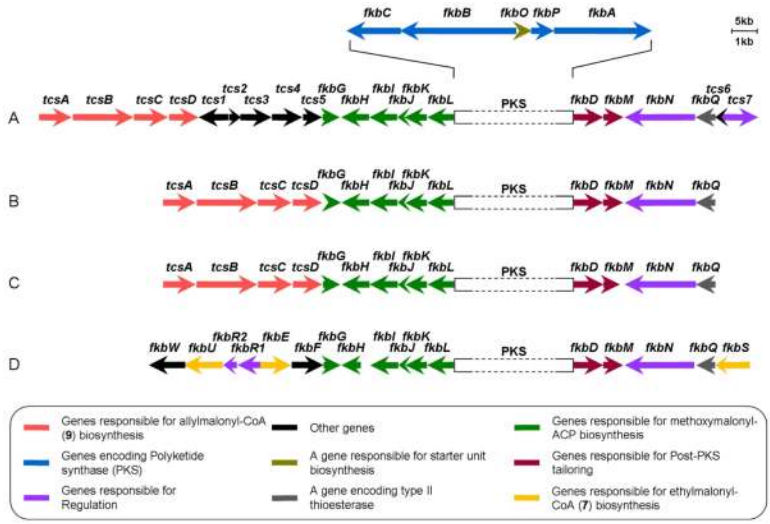

Figure 2. Organization of FK506 and FK520 biosynthetic gene clusters

(**A**) FK506 (**1**) biosynthetic gene cluster from *Streptomyces* sp. KCTC11604BP. (**B**) **1** cluster from *Streptomyces* sp. ATCC55098 (MA6858). (**C**) **1** cluster from *Streptomyces kanamyceticus* KCTC9225. (**D**) FK520 (**2**) cluster from *S. hygroscopicus* var. *ascomyceticus* ATCC14891.

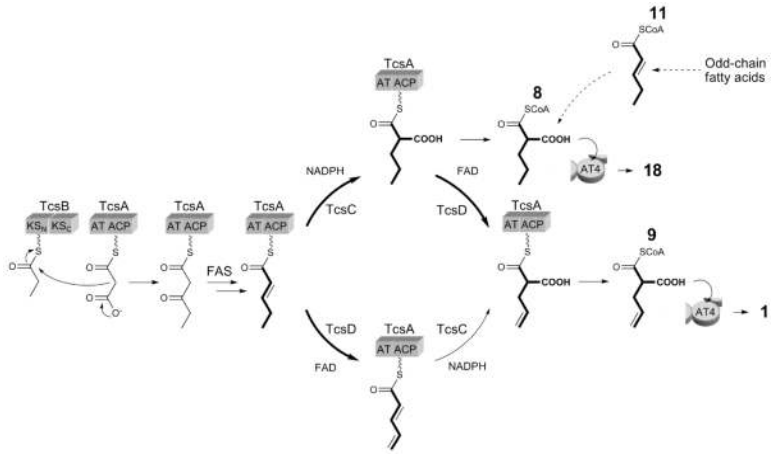

Figure 3. Proposed biosynthetic pathway of allylmalonyl-CoA as a novel five-carbon extender unit for FK506 PKS

The functions of four proteins, TcsA, TcsB, TcsC and TcsD, are deduced to be, respectively: an acyl transferase (AT) and acyl carrier protein (ACP) complex, a β-keto-acyl synthase (KS), a 2-pentenoyl-ACP carboxylase/reductase, and an acyl-ACP dehydrogenase. Bold lines indicate the biosynthetic steps characterized *in vitro*. Propylmalonyl- and allylmalonyl-ACPs are presumed to be converted to **8** and **9**, respectively, by an unidentified ACP:CoA transacylase-like enzyme. The previously proposed biosynthetic pathway to **8** from odd-chain fatty acids is shown in dashed lines. KS_N and KS_C represent the N-terminal and C-terminal KS domains of TcsB, respectively.

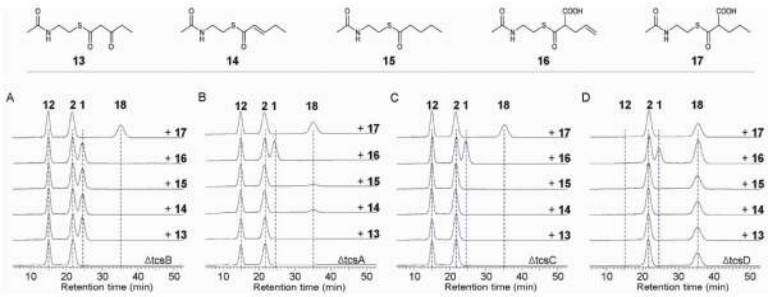

Figure 4. Chemical complementation of four different *tcs* deletion mutants with a variety of acyl-SNAC thioesters

(**A**) Chromatograms obtained from a culture of the ΔtcsB strain supplemented with 3-oxopentanoyl-SNAC (**13**), *trans*-2-pentenyl-SNAC (**14**), pentanoyl-SNAC (**15**), allylmalonyl-SNAC (**16**) or propylmalonyl-SNAC (**17**). (**B**) Chromatograms obtained from a culture of the ΔtcsA strain supplemented with **13**, **14**, **15**, **16** or **17**. (**C**) Chromatograms obtained from a culture of the ΔtcsC strain supplemented with **13**, **14**, **15**, **16** or **17**. (**D**) Chromatograms obtained from a culture of the ΔtcsD strain supplemented **13**, **14**, **15**, **16** or **17**. The structures of compounds **13**, **14**, **15**, **16** or **17** are shown in the upper panel.

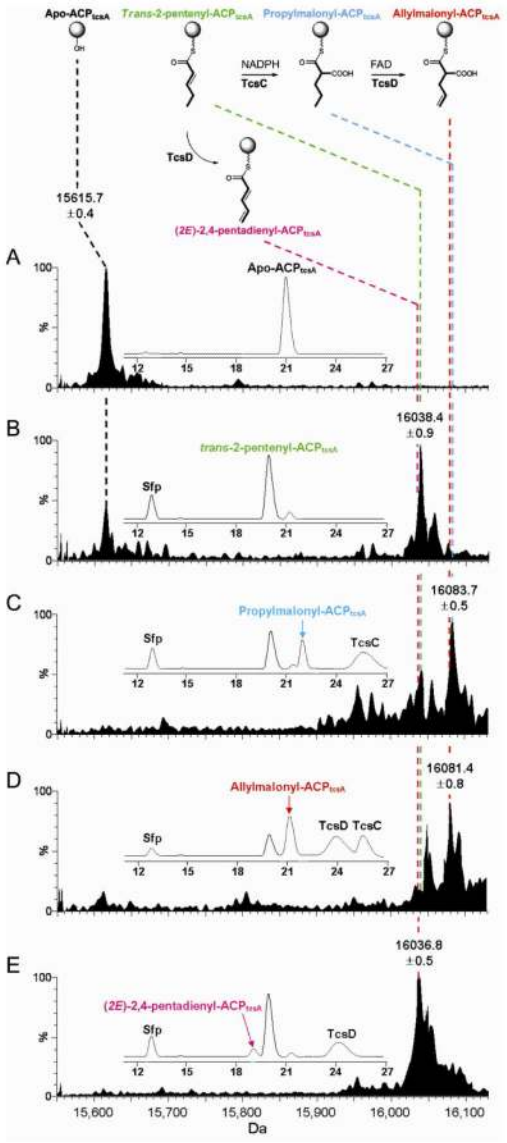

Figure 5. ESI-MS spectra of the biosynthetic intermediates during allylmalonyl-ACP$_{tcsA}$ biosynthesis

The top of the figure illustrates acyl-ACP$_{tcsA}$ intermediates of interest. From left to right: apo-ACP$_{tcsA}$, *trans*-2-pentenyl-ACP$_{tcsA}$ (+ 422 Da), (*2E*)-2,4-pentadienyl-ACP$_{tcsA}$ (+ 420 Da), propylmalonyl-ACP$_{tcsA}$ (+ 468 Da), and allylmalonyl-ACP$_{tcsA}$ (+ 466 Da). (**A**) Apo-ACP$_{tcsA}$ (calculated mass: 15,615.85 Da, assuming the N-terminal methionine residue is removed). (**B**) *Trans*-2-pentenyl-ACP$_{tcsA}$ (calculated mass: 16,037.95 Da). (**C**) Propylmalonyl-ACP$_{tcsA}$ (calculated mass: 16,083.98 Da). (**D**) Allymalonyl-ACP$_{tcsA}$ (calculated mass: 16,081.78 Da). (**E**) (*2E*)-2,4-pentadienyl-ACP$_{tcsA}$ (calculated mass: 16,035.75 Da). The insets in (**A**) to (**E**) depict the representative HPLC traces of each reaction mixture.

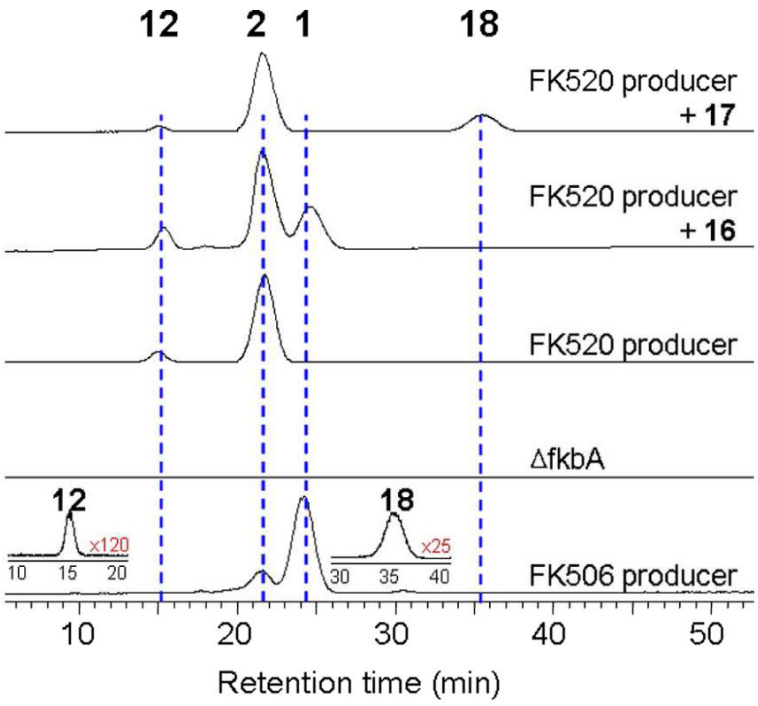

Figure 6. Chemical complementation study of an FK520 producer

HPLC-ESI-MS/MS chromatograms obtained from a culture of the wild-type strain *Streptomyces* sp. KCTC 11604BP, the ΔfkbA strain, and the FK520 (**2**) producer *S. hygroscopicus* var. *ascomyceticus* ATCC 14891 supplemented with allylmalonyl-SNAC (**16**) or propylmalonyl-SNAC (**17**).

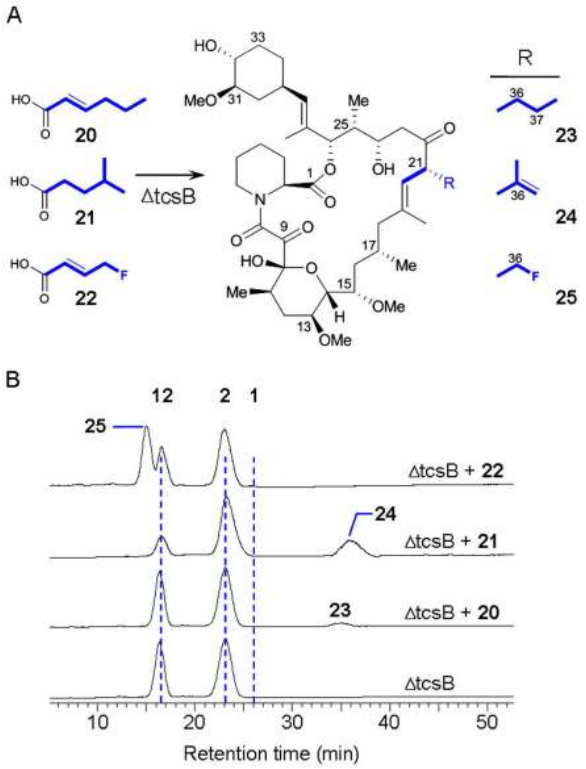

Figure 7. Generation of novel FK506 analogs through mutasynthesis

(**A**) Chemical structure of the FK506 analogs 36,37-dihydro-37-methyl-FK506 (**23**), 36-methyl-FK506 (**24**) and 36-fluoro-FK520 (**25**). (**B**) HPLC-ESI-MS/MS chromatograms obtained from the culture of ΔtcsB strain, separately supplemented with *trans*-2-hexenoic acid (**20**), 4-methylpentanoic acid (**21**), or 4-fluorocrotonic acid (**22**).

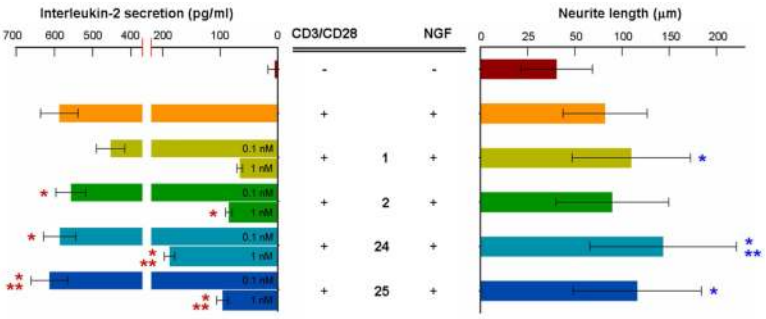

Figure 8. Immunosuppressive and neurite outgrowth activities of the FK506 analogs

Immunosuppressive properties of 36-methyl-FK506 (**24**) and 36-fluoro-FK520 (**25**), were compared with those of authentic FK506 (**1**) and FK520 (**2**) by quantification of interleukin (IL)-2 secreted from CD3/CD28-stimulated human T lymphocytes (left). **P* < 0.001 as compared with **1**-treated samples at the same concentration; ***P* < 0.001 as compared with **2**-treated samples at the identical concentration. Nerve regenerative properties of **24** and **25** were compared with those of **1** and **2** by measuring the neurite lengths of the nerve growth factor (NGF)-activated human neuroblastoma cell line SH-SY5Y (right). **P* < 0.001 as compared with NGF-treated samples; ***P* < 0.001 as compared with **1**-treated samples.