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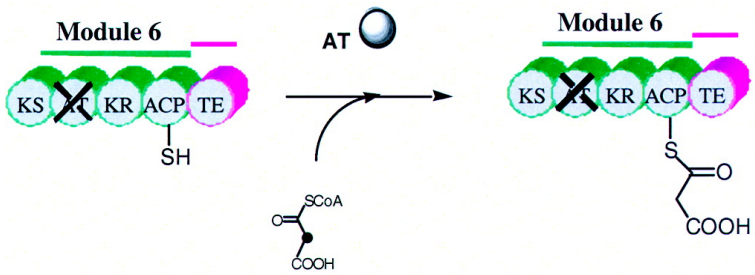

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Enhancing the Modularity of the Modular Polyketide Synthases: Transacylation in Modular Polyketide Synthases Catalyzed by Malonyl-CoA:ACP Transacylase

Pawan Kumar,[‡] Andrew T. Koppisch,[‡] David E. Cane,[§] and Chaitan Khosla*[,‡,,||,⊥]

Contribution from the Departments of Chemical Engineering, Chemistry, and Biochemistry, Stanford University, Stanford, California 94305, and Department of Chemistry, Brown University, Providence, Rhode Island 02912

Received July 21, 2003; E-mail: ck@chemeng.stanford.edu

Abstract: Selective incorporation of extender units in modular polyketide synthases is primarily controlled by acyl transferase (AT) domains. The AT domains catalyze transacylation of the extender unit from acyl-CoA to the phosphopantetheine arm of an acyl carrier protein (ACP) domain in the same module. New methods that can modulate the extender unit specificity of individual modules with minimal structural or kinetic perturbations in the engineered module are desirable for the efficient biosynthesis of novel natural product analogues. We have demonstrated that transacylation of malonyl groups onto an AT-null form of a mutant modular polyketide synthase by malonyl-CoA:ACP transacylase is an effective strategy for the engineered biosynthesis of site specifically modified polyketides. Using this strategy, 6-deoxyerythronolide B synthase was engineered to exclusively produce 2-desmethyl-6-deoxyerythronolide B. The productivity of the modified system was comparable to that of the wild-type synthase in vitro and in vivo.

Introduction

Modular polyketide synthases (PKSs) are multifunctional enzymes that catalyze the biosynthesis of many medicinally important natural products.[1,2] Domains responsible for individual catalytic steps in the biosynthesis of these compounds are organized in a modular fashion (Figure 1A).[3,4] The carbon backbones of polyketides are synthesized through repeated condensations between acyl-CoA-derived building blocks, typically malonyl-, methylmalonyl-, ethylmalonyl-, or methoxymalonyl-CoA. Selective incorporation of this range of extender units is partially responsible for the observed chemical diversity,[5,6] and is primarily controlled by acyl transferase (AT) domains that catalyze transacylation of the extender unit from acyl-CoA to the phosphopantetheine arm of the acyl carrier protein (ACP) domain in the same module. In the last several years, two strategies have emerged for regioselective substitution of a natural extender unit with an unnatural building block: genetic substitution of AT domains within modules[7−11] and site-directed mutagenesis of endogenous AT domains.[12] In most cases where entire AT domains were replaced, the resultant PKSs were observed to have a significantly attenuated catalytic efficiency.[7−11,13] Similarly, site-directed mutagenesis is limited in that the specificity of a natural AT domain may only be relaxed, thereby allowing the incorporation of both unnatural and natural extender units.[12] Thus, new methods that can predictively modulate the extender unit specificity of individual modules with minimal structural or kinetic perturbations in the engineered module are desirable.

Recently, Shen and co-workers reported the isolation of an unusual polyketide gene cluster encoding leinamycin (Lnm) biosynthesis.[14] Individual modules of the Lnm PKS lack their own AT domains. Instead, they use an exogenous malonyl-CoA-specific transacylase for transferring malonyl extender units onto each ACP domain within the multimodular PKS. Motivated by the apparent efficiency of this novel architectural principle, here we report a new strategy for regiospecific alteration of the extender unit sequence in a polyketide backbone. Our strategy

* To whom correspondence should be addressed.
‡ Department of Chemical Engineering, Stanford University.
§ Department of Chemistry, Brown University.
|| Department of Chemistry, Stanford University.
⊥ Department of Biochemistry, Stanford University.

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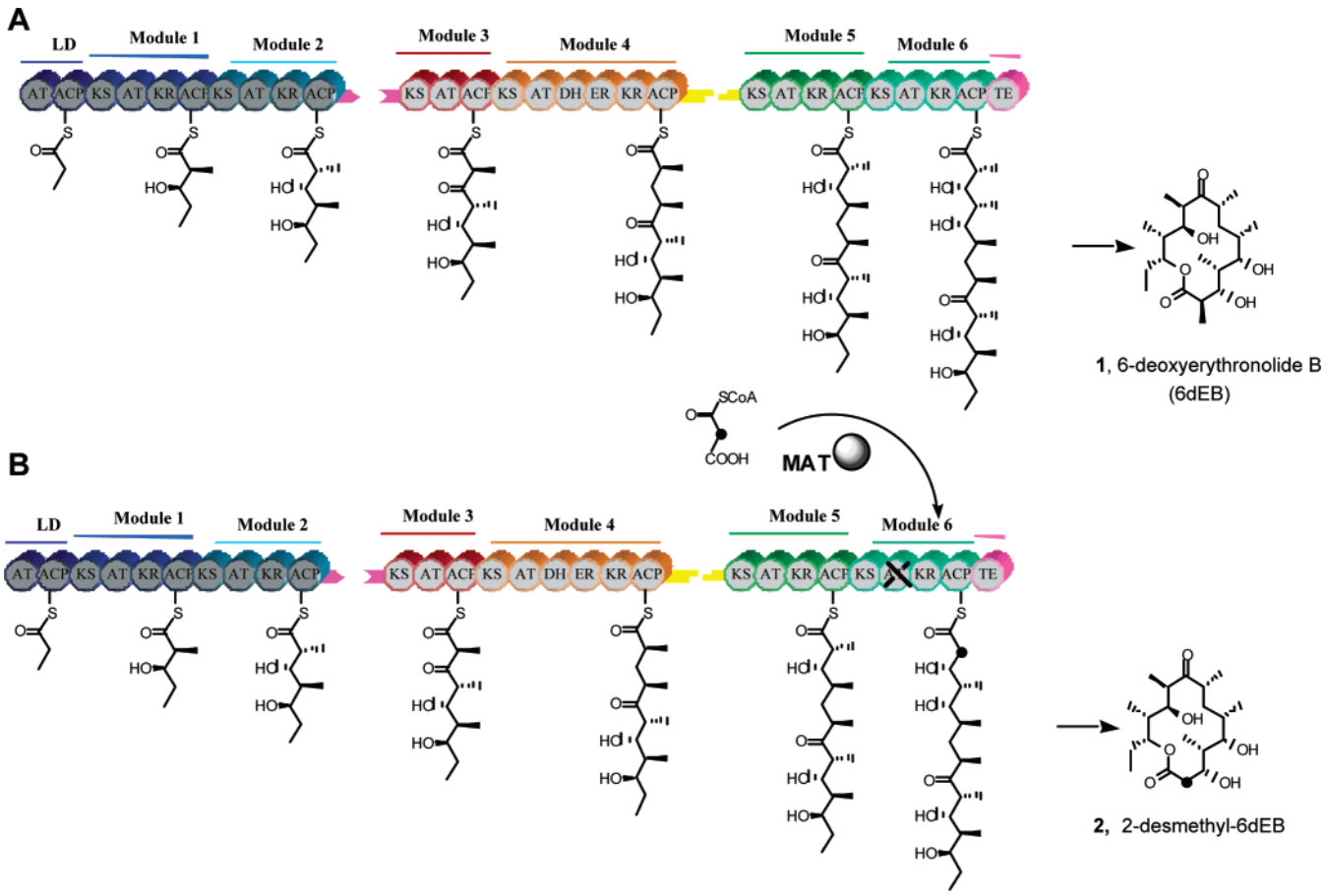

Figure 1. (A) Schematic diagram of the biosynthesis of 6dEB (**1**) by 6-deoxyerythronolide B synthase. Each polypeptide, DEBS1, DEBS2, and DEBS3, contains two modules, and each module comprises a set of active site domains responsible for addition and modification of an extender unit. (B) Proposed strategy for the biosynthesis of 2-desmethyl-6dEB (**2**). AT6 on DEBS3 is inactivated by site-directed mutagenesis. An exogenous transacylase provides the unnatural malonyl extender unit to the megasynthase at ACP6.

utilizes an exogenous transacylase for loading extender units onto a modular polyketide synthase whose intrinsic transacylase activity is inactivated by a single Ser → Ala mutation (Figure 1B). The effectiveness of this strategy was evaluated by protein labeling procedures, multiple turnover analysis, and eventually in vivo biosynthesis of a product containing an unnatural building block. The observation of robust turnover of polyketides in vitro and the observation of comparable biosynthetic yields of macrolactone by the wild-type and mutant PKSs are strong indicators of the feasibility of this strategy.

Results

Proposed Strategy. In general, modular PKSs with intact AT domains exhibit stringent selectivity for extender units. Recently, we have shown that the AT domain selectivity for a given acyl-CoA is apparently the only checkpoint for extender unit specificity of a PKS module.[13] We therefore hypothesized that an exogenous acyl transferase could complement a mutant PKS module whose AT domain had been inactivated via site-directed mutagenesis. The *Streptomyces coelicolor* malonyl-CoA:ACP transacylase (MAT)[15] was chosen as an exogenous transacylase for our studies, because of its high turnover rates (~100 s^{-1} at 0 °C)[16] and its tolerance for a broad range of ACPs.[17] To test this hypothesis, a series of experiments, described below, were performed.

Labeling of Modules. The terminal module of the 6-deoxyerythronolide B synthase (DEBS) (designated M6+TE due to the presence of a covalently attached thioesterase domain) was chosen as the initial target for our studies. This wild-type module and an AT6-null mutant (designated M6+TE(S2107A)) were expressed in *E. coli*, purified, and analyzed for their ability to be transacylated with malonyl extender units by the *S. coelicolor* MAT. Protein labeling assays were performed in the presence of a mixture of 100 μM [2-^{14}C]malonyl-CoA (the preferred substrate of the *S. coelicolor* MAT) and unlabeled methylmalonyl-CoA (the preferred substrate of AT6). M6+TE (S2107A), but not the wild-type M6+TE, was efficiently radiolabeled in 10 min, as judged by SDS−PAGE radiography (Figure 2).

In Vitro Turnover by the Engineered System. The turnover efficiency of the mutant module was measured by incubating M6+TE(S2107A) with MAT and a suitable electrophilic substrate for this module, the *N*-acetylcysteamine thioester of (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid. Although this substrate is the natural substrate of the second module of DEBS (designated M2+TE), it is recognized and elongated with comparable kinetic efficiency by both modules.[13] The reaction was initiated by the addition of 100 μM [2-^{14}C]malonyl-CoA.

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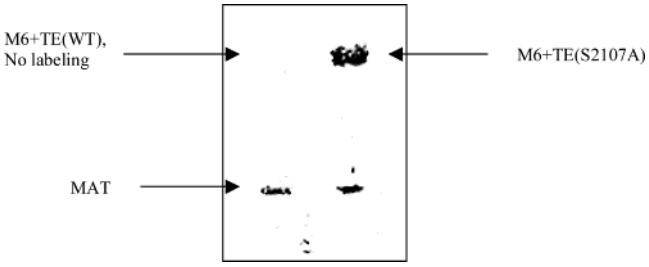


Figure 2. *In-trans* labeling of modules: lane 1, wild-type M6+TE; lane 2, M6+TE(S2107A). The modules (5 μM) were incubated with 1 μM MAT, 100 μM [2-^{14}C]malonyl-CoA, and 200 μM unlabeled methylmalonyl-CoA.

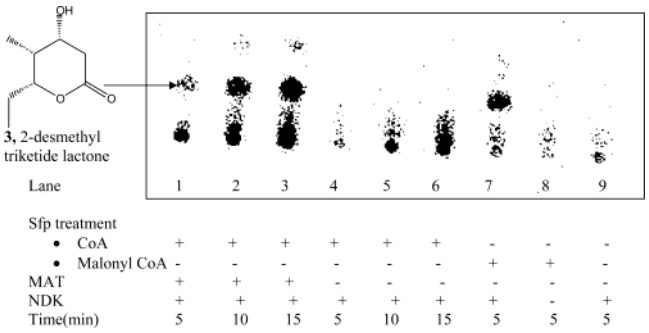


Figure 3. Radio TLC analysis of MAT-assisted turnover of DEBS module 6+TE. Apo-M6+TE(S2107A) was first treated with *sfp* in the presence of CoASH (or [2-^{14}C]malonyl-CoA as a single-turnover reference). Various controls exhibit the essential role of the *S. coelicolor* MAT in the turnover of this module.

Under these conditions the MAT rapidly hydrolyzes malonyl-CoA in addition to catalyzing malonyl-CoA:ACP transacylation; consequently, the reaction mixture was periodically replenished with malonyl-CoA. Multiple equivalents of the expected 2-desmethyl triketide lactone product **3** were synthesized, with each equivalent being released from the enzyme in under 5 min (Figure 3). From this it can be concluded that the turnover number of M6+TE(S2107A) in the presence of exogenous MAT is >0.2 min^{-1}. This parameter is comparable to the turnover number of wild-type M6+TE in the presence of similar concentrations of the same electrophilic substrate.[13]

An ACP from a Modular PKS as a Substrate for the *S. coelicolor MAT*. To quantify the ability of the MAT to transacylate ACP domains from modular PKSs, we adapted an assay used in our laboratory to examine MAT-catalyzed transacylation of malonyl building blocks onto ACPs from type II PKSs.[16] For this purpose the ACP domain from module 2 of DEBS (designated ACP2) was expressed as a stand-alone holoprotein and purified to homogeneity. Although we could not observe saturation of the MAT with ACP2, the k_{cat}/K_m was estimated to be $(2.1 \pm 0.4) \times 10^{-4}$ s^{-1} μM^{-1} by the linear fitting of the v vs [ACP2] plot (Figure 4).

Biosynthesis and Characterization of 2-Desmethyl-6dEB. The engineered *E. coli* strain BAP1 (see the Experimental Section for details) was transformed with plasmids expressing either wild-type DEBS or a variant carrying the M6+TE-(S2107A) mutation. Two analogous cell lines in which the MAT was coexpressed with each PKS were also prepared. As expected, transformants expressing the wild-type DEBS, with or without the *S. coelicolor* MAT, exclusively produced 6-deoxyerythronolide B (6dEB). The identity of 6dEB was confirmed by LC−MS (Table 1) and by comparison with an

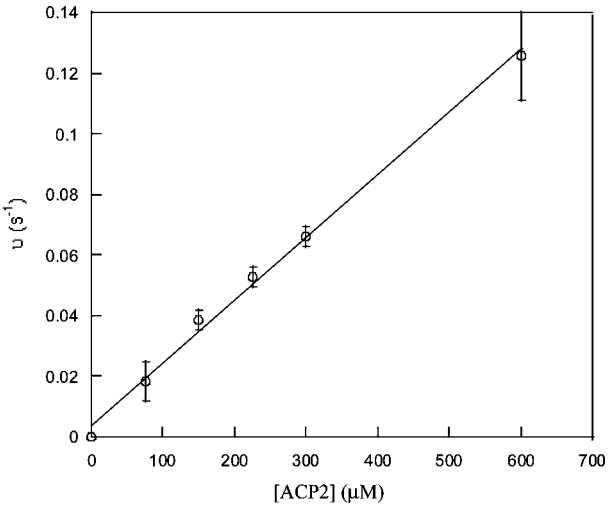
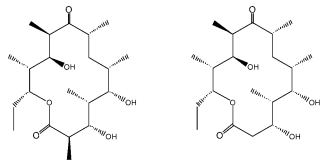


Figure 4. A typical initial rate (s^{-1}) vs [ACP2] (μM) plot. The concentration of MAT used was approximately 100 nM, and malonyl-CoA was held constant at 300 μM. Initial rates were determined on ice in duplicate, and k_{cat}/K_m was determined from a linear fitting of the data.

Table 1. Masses of Signature Ions Observed from the Extracts of Various Systems upon [^{12}C/^{13}C]Propionic Acid Feeding[a]



1 **2**

6DEB, M=386 2-desmethyl-6DEB, M=372

	mass observed			
construct[b]	$M + H^+$	$[M - H_2O] + H^+$	$[M - 2H_2O] + H^+$	compd
A	387/394	369/376	351/358	**1**
B	387/394	369/376	351/358	**1**
C	373/379	355/361	337/343	**2**
D	373/379	355/361	337/343	**2**
E	373/379	355/361	337/343	**2**

[a] All masses are observed at approximately the same retention time (17 min for 2-desmethyl-6DEB and 18 min for 6DEB) on a C_{18} column. M + 7 and M + 6 increases upon feeding ^{13}C-labeled propionic acid are signatures for 6DEB and 2-desmethyl-6DEB, respectively. Both macrolides yield M − 18 and M − 36 ions due to the loss of one and two water molecules. The reversed-phase HPLC retention time of compounds produced by constructs D and E was identical to the retention time of authentic 2-desmethyl-6DEB produced by construct C. [b] Construct A expresses wild-type DEBS. Construct B coexpresses wild-type DEBS and the *S. coelicolor* MAT enzyme. Construct C expresses a derivative of wild-type DEBS in which AT6 has been replaced by a malonyl-specific AT domain. Construct D expresses DEBS(AT6^0). Construct E coexpresses DEBS(AT6^0) and the *S. coelicolor* MAT enzyme. For a description of the constructs, see the section titled "Biosynthesis of 2-Desmethyl-6dEB".

authentic sample. Surprisingly, a small amount of a 6dEB-like compound was produced by the strain expressing the DEBS-(AT6^0) mutant. LC−MS analysis suggested that the compound was a desmethyl derivative of 6dEB (Table 1). To confirm this hypothesis, [1-^{13}C]propionate was fed to this strain of *E. coli* in lieu of unlabeled propionate. (All C_3 building blocks for 6dEB biosynthesis in *E. coli* are derived from exogenous propionate.)[18] LC−MS analysis revealed a compound with a retention time

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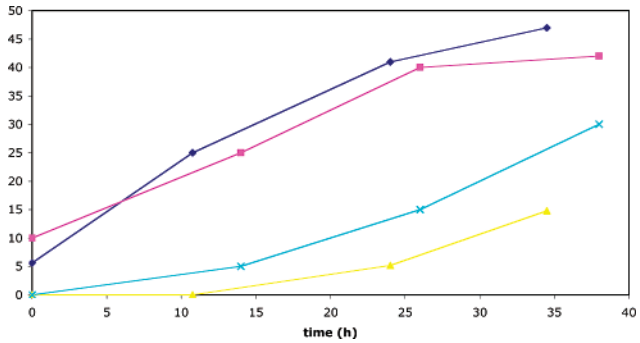

Figure 5. Fed-batch fermentation of recombinant *E. coli* strains producing 6dEB (wild-type DEBS system, construct A) and 2-desmethyl-6dEB (AT6-null plus *S. coelicolor* MAT system, construct E). Both OD_{600} and product titers were monitored for 36 h postinduction with IPTG, as described in the Experimental Section. See Table 1 for the definition of constructs (■, construct A, OD_{600}; ◆, construct E, OD_{600}; ×, construct A, 6dEB titer, mg/L; ▲, construct E, 2-desmethyl-6dEB titer, mg/L).

identical with that of the previously observed compound, but with an MW of 378, consistent with the incorporation of six propionate equivalents into a desmethyl-6dEB compound. The same compound was also produced by the strain in which DEBS($AT6^0$) was coexpressed with *S. coelicolor* MAT. The identity of this compound as 2-desmethyl-6dEB was confirmed by comparison with an authentic reference compound, isolated from a variant of DEBS in which AT6 was replaced by the malonyl-specific AT2 domain from the rapamycin PKS.[19]

The polyketide productivity of all these strains was evaluated under fed-batch fermentation conditions, performed under conditions described earlier.[20] The productivity of the DEBS-($AT6^0$) mutant in the presence of *S. coelicolor* MAT was comparable to that of wild-type DEBS, as analyzed by an ELSD (evaporative light scattering detector) coupled with HPLC (~0.5 mg L^{-1} h^{-1} versus ~0.7 mg L^{-1} h^{-1}) (Figure 5).

Discussion

Although the ACP domains in PKS modules are typically viewed as an integral part of a larger cluster of domains, they can also be recognized and acted upon by other proteins. For example, they undergo posttranslational phosphopantetheinylation, catalyzed by a stand-alone phosphopantetheinyl transferase (PPTase; Sfp in *E. coli* BAP1).[18,21] The recently reported leinamycin synthase further expands the roster of stand-alone proteins that can access ACP domains to include an acyltransferase.[14] Indeed, it has been suggested that PPTases and acyltransferases share a common helical flap for recognition of acyl carrier proteins.[22] We therefore hypothesized that a stand-alone acyltransferase from a type II PKS or fatty acid synthase may be able to charge an ACP domain in a PKS module whose endogenous AT domain had been inactivated. Although stand-alone MAT proteins are present in all bacteria, they are unable to catalyze the same reaction on the ACP domain of a wild-type PKS module, presumably because of the intrinsic editing activity of AT domains.[5] To test this hypothesis, we inactivated the AT domain in M6+TE of DEBS by replacing the active site serine with an alanine residue.[13] Radiolabeling studies in the presence of MAT and [2-^{14}C]malonyl-CoA revealed that the wild-type module could not be labeled, but the AT-null mutant could be efficiently labeled by malonyl-CoA.

Previous studies[13] showed that the chain elongation reaction catalyzed by individual PKS modules is tolerant toward unnatural extender units, suggesting that the substrate specificity of the AT domain is the primary checkpoint for nucleophile recognition and turnover by this module. Consistent with this proposal, the turnover number of the M6+TE(S2107A) mutant using malonyl-CoA and exogenous MAT is comparable to that of wild-type M6+TE (~0.1−1 min^{-1}) under otherwise equivalent conditions.[13] Comparable efficiency was also observed for the M2+TE(S2598A) mutant in a similar in vitro assay, suggesting that MAT-mediated transacylation of malonyl extender units onto modular PKSs is efficient and general.

The efficiency of this alternative strategy of biosynthetic engineering of complex polyketides is presumably dictated by the kinetics of malonyl transfer by the *S. coelicolor* MAT onto the modular ACP. To quantify the catalytic efficiency of this process, holo-ACP from DEBS module 2 (ACP2) was expressed as an intact protein[23] and subjected to quantitative kinetic analysis. A k_{cat}/K_m of $(2.1 \pm 0.4) \times 10^{-4}$ s^{-1} μM^{-1} was observed for ACP2. This is significantly lower than the k_{cat}/K_m for a preferred ACP substrate of the *S. coelicolor* MAT (~1 s^{-1} μM^{-1}).[16] However, it compares well to the kinetics of acyl-CoA:ACP transacylation by AT domains of modular PKSs[6] and of transacylation catalyzed by the stand-alone AT enzyme in the Lnm PKS.[14]

To compare the in vivo efficiency of this method, the *S. coelicolor* MAT was cotransformed with the AT6-null mutant of DEBS into *E. coli* BAP1.[18] As controls, *E. coli* strains containing (i) the AT6-null mutant without the *S. coelicolor* MAT gene and (ii) a variant of DEBS in which AT6 was replaced by the malonyl-specific AT2 domain from the rapamycin PKS[19] were used. Each strain produced 2-desmethyl-6dEB, the expected polyketide analogue. These findings suggested that, in addition to the *S. coelicolor* MAT, the endogenous malonyl-CoA:ACP transacylase in *E. coli* was also able to catalyze transacylation of a malonyl extender unit onto the "vacant" ACP domain. In fed-batch fermentations, the productivity of the AT6-null mutant cotransformed with *S. coelicolor* MAT was comparable to that of the wild-type DEBS. In contrast, earlier studies have shown that AT domain substitution results in a significant attenuation of polyketide productivity.[13,19]

In conclusion, we have demonstrated that transacylation of an AT-null form of a mutant modular PKS by a coexpressed stand-alone AT enzyme is an effective strategy for the engineered biosynthesis of site specifically modified polyketides. All MATs and other stand-alone AT enzymes identified to date are malonyl-specific; hence, the range of substrates that can currently be introduced by this route is limited to malonyl-CoA. However, guided by the recent X-ray crystal structure of the *S. coelicolor* MAT,[22] it may be possible to engineer this enzyme to accept other unnatural acyl-CoA substrates. Similarly, the specificity of this MAT toward selected ACP domains from a target modular PKS may also be improved via protein engineer-

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ing. The principal advantage of this method is its ability to leave intact the easily perturbed and as-yet-uncharacterized tertiary and quaternary structure of a modular PKS.

Materials and Methods

Reagents and Chemicals. [2-^{14}C]Malonyl-CoA (55 mCi/mmol) was purchased from ARC radiochemicals. [1-^{13}C]Sodium propionate was from Sigma. All other chemicals were from Sigma or Fluka (St. Louis, MO). The plasmids pET21c and pET28c were from Novagen. Ni-NTA affinity resin was from Qiagen Inc. TLC plates were obtained from J. T. Baker (Phillipsburg, NJ).

Plasmids. M2+TE, M2+TE(S2598A) [or M2+TE(AT2^0)], M6+TE, and M6+TE(S2107A) [or M6+TE(AT6^0)] are encoded by pRSG64, pMH28, pRSG54, and pMH21 as described recently.[13] MAT is encoded by pGFL16, which contains the MAT cassette on a pET21 vector and expresses an N-terminal hexahistidine tagged protein. MAT was also constructed on a chloramphenicol-resistant vector by replacing the TEII cassette with the MAT cassette on pBP190[20] between the *Xba*I and *Hin*dIII sites to yield pPK201. DEBS3+TE(AT6^0) was obtained by replacing M6+TE on pBP129 with M6+TE(AT6^0) from pMH21 between *Bsa*BI and *Hin*dIII, yielding pPK202. A plasmid pPK203 expressing both DEBS2 and DEBS3+TE(AT6^0) was then obtained in the same fashion as pBP130.[18] Propionyl-CoA carboxylase and DEBS1 are expressed by pBP144.[18] pBP130 expresses wild-type DEBS2 and DEBS3+TE.[18] Plasmid pBP173 is a derivative of pBP130 and pJL409 which expresses DEBS2 and DEBS3+TE where AT6 is replaced by malonyl-CoA-specific AT2 from the rapamycin PKS.[19]

Bacterial Strains. Expression of the apoproteins and MAT was achieved by using the above plasmids in *E. coli* BL21(DE3). For in vivo feeding experiments, and for the expression of holoproteins, BAP1, which is an engineered *E. coli* BL21(DE3) strain, was used.[18] BAP1 contains a chromosomally integrated copy of the *sfp* phosphopantetheinyl transferase from *Bacillus subtilis*, which is required for the posttranslational phosphopantetheinylation of the ACP domains of individual modules. The cells were transformed by electroporation and selected over LB plates with appropriate resistance(s).

Expression and Purification of MAT. The MAT protein was expressed and purified as described previously using pGFL16.[16,24]

Expression and Purification of DEBS ACP2. The plasmid containing the DEBS ACP2 (pNW7) was introduced into host strain BAP1. The ACP2 was expressed in a manner similar to that of MAT, with postinduction growth carried out at 22 °C for 4−5 h. After being harvested by centrifugation (4000 rpm, 20 min), the cells were stored at −80 °C until use.

Frozen cells were lysed, and the ACP2 was purified in a fashion (Ni-NTA agarose/Hi-trap Q) identical with that for MAT. Generally, the ACP2 is greater than 98% pure after elution from the Ni-NTA agarose (as judged by SDS−PAGE). Any minor contaminants which remain after this step are removed by the anion exchange purification. This procedure provides large quantities of ACP2 which are free of any contaminating proteins which may be reactive toward it.

Expression and Purification of M2+TE, M2+TE(S2598A), M6+TE, and M6+TE(S2107A). Apo and holo modules were purified from BL21 and BAP1 cells, respectively, as described previously.[13]

Assay for MAT:ACP2 Transacylation. All assays were performed in buffer G (100 mM $NaHPO_4$, 2 mM EDTA, 2 mM DTT, pH 6.8) supplemented with 1 mg/mL BSA to ensure protein stability at low concentrations. In every case, ACP2 and malonyl-CoA were added to the solution and placed on ice, and the reaction was initiated with the addition of the MAT (typically ∼100 nM). Malonyl-CoA was held constant at a saturating concentration (300 μM), while ACP2 concentrations were varied between 50 and 600 μM. Aliquots of 7 μL of the reaction mixture were removed at appropriate time points and quenched into 30 μL of ice cold 30% TCA in an Eppendorf tube. BSA (30 μL of a 10 mg/mL stock solution) was added as a protein carrier, and the ACP was allowed to precipitate at 0 °C for 20 min. The precipitated protein was centrifuged (13000*g*, 5 min), and the supernatant was removed. A 600 μL sample of ice cold 10% TCA was then added, and the solution was vortexed and centrifuged (13000*g*, 5 min). After the TCA solution was decanted, the protein was dissolved in 100 μL of 98% formic acid for 15 min. The protein solution was placed in a scintillation vial, and the Eppendorf tube was washed with 100 μL of water, which was also added to the vial. A 5 mL of Ready-Safe scintillation fluid was added, and the sample was vortexed. The extent of isotopic incorporation was then estimated by liquid scintillation counting.

Initial rate (below 5% conversion) data obtained from these experiments were used to calculate k_{cat}/K_M. All analyses were performed in duplicate at six different concentrations of ACP2.

Labeling of Modules. A 5 μM concentration of holo-PKS modules was incubated with 100 μM [2-^{14}C]malonyl-CoA, 200 μM unlabeled methylmalonyl-CoA, and 1 μM MAT in 100 mM sodium phosphate (pH 7.2), 1 mM EDTA, and 20% glycerol at 0 °C. After 10 min the reaction was quenched by adding SDS−PAGE loading buffer. Proteins were denatured by heating at 70 °C for 5 min and loaded onto an SDS−PAGE gel. The gel was dried and analyzed by phosphoimaging (Figure 2).

Turnover of an AT^0 Mutant PKS Module Supported by MAT-Catalyzed Transacylation. Apo modules were converted into the holo form in vitro as described before.[13] A 30 μM concentration of M2+TE-(S2598A) or M6+TE(S2107A) was incubated with 0.1 μM Sfp and 100 μM CoASH (or [2-^{14}C]malonyl-CoA for single-turnover reactions) in 100 mM sodium phosphate (pH 6.6), 2 mM DTT, 1 mM EDTA, and 10 mM $MgCl_2$ at 30 °C for 30 min. Subsequently, 35 μL of the above mixture was adjusted to 400 mM sodium phosphate, pH 7.2, 2 mM DTT, 1 mM EDTA, and 20% glycerol. A 5 mM concentration of NDK, 4 mM NADPH, and 1 μM MAT were also added. The chain elongation reaction was initiated by adding 100 μM [^{14}C]malonyl-CoA in a final volume of 70 μL at 30 °C. A 20 μL sample of the reaction was withdrawn and quenched by mixing with 300 μL of ethyl acetate every 5 min, and at the same time malonyl-CoA was replenished in the balance reaction to maintain a 100 μM total concentration. Single-turnover reactions were carried out without MAT and malonyl-CoA under the same conditions as described above. Products were extracted twice by 300 μL of ethyl acetate and analyzed by thin-layer chromatography as previously described.[13] A typical radio TLC image is shown in Figure 3.

Biosynthesis of 2-Desmethyl-6dEB. The protocol for biosynthesis of macrolactones used was similar to that previously described.[18] BAP1 cells were transformed with pBP144/pBP130, pBP144/pBP130/pPK201, pBP144/pBP173, pBP144/pPK203, or pBP144/pPK203/pPK201, yielding constructs A, B, C, D, and E, respectively. Constructs A, C, and D were selected on plates containing kanamycin (50 μg/mL) and ampicillin (100 μg/mL). Constructs B and E were selected over plates containing kanamycin (50 μg/mL), ampicillin (100 μg/mL), and chloramphenicol (30 μg/mL). After overnight growth, starter cultures were grown in 5 mL of LB containing appropriate antibiotics for 6 h at 37 °C. The cells were harvested and used to inoculate 2 × 100 mL of LB in two 500 mL flasks for each system. The flasks were shaken at 250 rpm at 37 °C till OD_{600} reached 0.6. The cultures were cooled to room temperature in a water bath, and IPTG was added to a final concentration of 100 μM to induce protein production. Unlabeled or ^{13}C-labeled propionic acid was separately fed into two flasks for each system to a final concentration of 250 mg/L. The flasks were shaken overnight at 22 °C at 250 rpm, and the cells were harvested and the products were extracted from the media using 200 mL of ethyl acetate twice. The products were dried in vacuo and analyzed by APCI LC−MS (Table 1).[19]

(24) Dreier, J.; Shah, A. N.; Khosla, C. *J. Biol. Chem.* **1999**, *274*, 25108−25112.

Fed-batch fermentations were also performed as previously described[20] to compare the productivity of the AT-null system with that of the wild-type system producing 6dEB. Samples were monitored for 36 h postinduction. After the cells were harvested from the samples, the products were extracted from 3.5 mL of broth with 5 mL of ethyl acetate twice. The products were then dried in vacuo and resuspended in 200 μL of water. A 25 μL sample of the above mixture was injected into an HPLC-coupled ELSD system for product quantification.[20] The cell density and product yields are plotted as a function of postinduction time in Figure 5.

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