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Assessing the Balance between Protein−Protein Interactions and Enzyme−Substrate Interactions in the Channeling of Intermediates between Polyketide Synthase Modules

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Abstract: 6-Deoxyerythronolide B synthase (DEBS) is the modular polyketide synthase (PKS) that catalyzes the biosynthesis of 6-deoxyerythronolide B (6-dEB), the aglycon precursor of the antibiotic erythromycin. The biosynthesis of 6-dEB exemplifies the extraordinary substrate- and stereo-selectivity of this family of multifunctional enzymes. Paradoxically, DEBS has been shown to be an attractive scaffold for combinatorial biosynthesis, indicating that its constituent modules are also very tolerant of unnatural substrates. By interrogating individual modules of DEBS with a panel of diketides activated as *N*-acetylcysteamine (NAC) thioesters, it was recently shown that individual modules have a marked ability to discriminate among certain diastereomeric diketides. However, since free NAC thioesters were used as substrates in these studies, the modules were primed by a diffusive process, which precluded involvement of the covalent, substrate-channeling mechanism by which enzyme-bound intermediates are directly transferred from one module to the next in a multimodular PKS. Recent evidence pointing to a pivotal role for protein−protein interactions in the substrate-channeling mechanism has prompted us to develop novel assays to reassess the steady-state kinetic parameters of individual DEBS modules when primed in a more "natural" channeling mode by the same panel of diketide substrates used earlier. Here we describe these assays and use them to quantify the kinetic benefit of linker-mediated substrate channeling in a modular PKS. This benefit can be substantial, especially for intrinsically poor substrates. Examples are presented where the k_{cat} of a module for a given diketide substrate increases >100-fold when the substrate is presented to the module in a channeling mode as opposed to a diffusive mode. However, the substrate specificity profiles for individual modules are conserved regardless of the mode of presentation. By highlighting how substrate channeling can allow PKS modules to effectively accept and process intrinsically poor substrates, these studies provide a rational basis for examining the enormous untapped potential for combinatorial biosynthesis via module rearrangement.

Introduction

Modular polyketide synthases (PKSs) are large, multidomain enzymes that catalyze the biosynthesis of secondary metabolites in bacteria. While numerous medicinally significant polyketide secondary metabolites—including the antibiotic erythromycin, the antitumor agent epothilone, and the immunosuppressant FK506—have been discovered, the great promise of PKSs is not the products that are naturally made in bacteria, but the novel and structurally diverse polyketides that could potentially be generated in a combinatorial fashion by engineered polyketide

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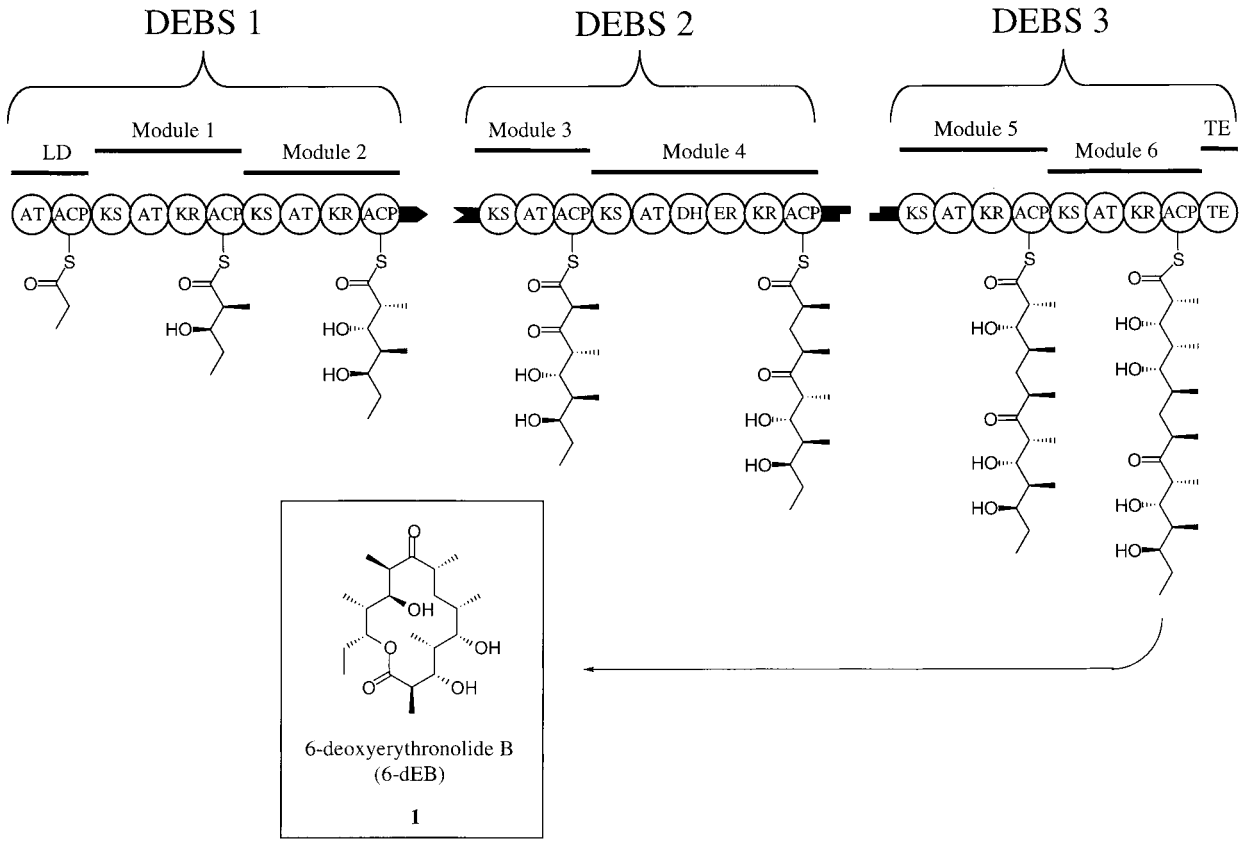

Figure 1. Schematic illustration of DEBS, its intermediates, and the 6-dEB final product. DEBS consists of three polypeptides—DEBS1, DEBS2, and DEBS3. Individual catalytic domains are represented by circles, and interpolypeptide linkers are shown as solid tabs between DEBS1 and DEBS2 and between DEBS2 and DEBS3. DEBS catalyzes the biosynthesis of 6-deoxyerythronolide B (6-dEB), the polyketide core of the antibiotic erythromycin, and comprises six individual modules. Each module contains three core catalytic domains—ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP)—as well as a variable number of optional domains—ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). In addition to the six modules, there is a loading didomain (LDD) at the N terminus of DEBS1 that is responsible for priming the synthase with a propionyl unit and a thioesterase (TE) domain at the C terminus of DEBS 3 that is responsible for chain release and macrocyclization. Polyketide biosynthesis proceeds in an assembly-line fashion such that the incoming polyketide chain is loaded onto the KS of an extending module from the ACP of the previous module. This is followed by a decarboxylative condensation reaction between the growing chain and a methylmalonyl-derived C_3 extender unit that has been loaded onto the ACP by the AT. This C−C bond-forming reaction places the growing chain on the ACP, where it can then undergo post-condensational modifications catalyzed by KR, DH, and ER before being passed to the KS of the downstream module.

synthases. This combinatorial potential of PKSs is derived from its unique modular architecture, which is exemplified in the prototypical model of this class of megasynthases—the 6-deoxyerythronolide B synthase (DEBS; Figure 1).

There are numerous proven strategies for rationally manipulating polyketide structure by engineering DEBS. For example, it has been demonstrated that DEBS is amenable to the introduction of unnatural side chains at the C_{13} and C_{11} positions via precursor-directed feeding of diketides,[1−3] as well as via replacement of loading didomains from alternative synthases.[4] In addition, protein engineering of DEBS can generate truncated polyketides,[5−8] epimerized polyketides,[9−12] desmethyl polyketides,[13−16] polyketides containing various degrees of modification of the β-keto groups,[17−21] and combinations thereof.[22] However, one approach for generating diversity in polyketides

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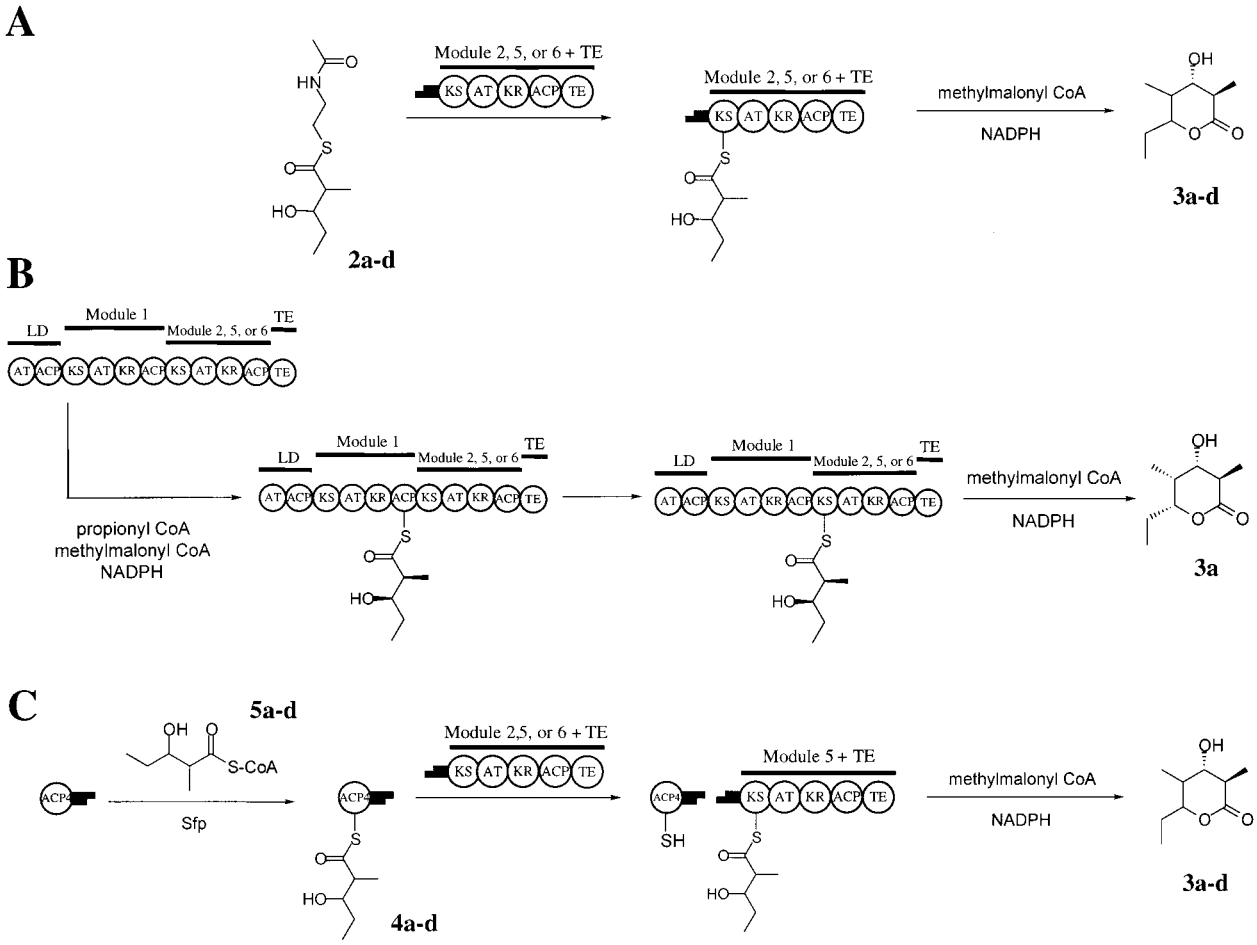

Figure 2. Schematic illustrations of the three mechanisms of loading a DEBS module with a diketide. (A) In a diffusive mechanism, diketides that have been activated as *N*-acetylcysteamine thioesters (diastereomers **2a**−**d**) are loaded exogenously onto the KS domain. Claisen-like condensation with a C_3-unit derived from methylmalonyl CoA followed by NADPH-dependent reduction gives the corresponding triketide lactone products (**3a**−**d**). (B) In an intrapolypeptide channeling mechanism, a diketide that is generated by module 1 from propionyl CoA, methylmalonyl CoA, and NADPH is passed intramolecularly from ACP1 to KS2. Subsequent elongation and reduction afford the triketide lactone **3a**. (C) In an interpolypeptide channeling mechanism, diketides that have been chemoenzymatically attached to ACP4 by Sfp (**4a**−**d**) are transferred to the KS domain on a separate polypeptide. Elongation and reduction afford the corresponding triketide lactones (**3a**−**d**). In all cases suffix "**a**" refers to the (2*S*,3*R*) diastereomer, suffix "**b**" refers to the (2*R*,3*S*) diastereomer, suffix "**c**" refers to the (2*S*,3*S*) diastereomer, and suffix "**d**" refers to the (2*R*,3*R*) diastereomer. See also Figure 3.

that has been exploited only to a limited extent[23,24] is the fusion of intact modules (or groups thereof) from different PKSs to generate chimeric assembly lines. While the application of such a strategy takes advantage of the natural catalytic grouping of the modules to produce enzymes of improved catalytic effectiveness, two major issues must be addressed to rationally implement a modular rearrangement strategy for combinatorial biosynthesis. First, the molecular recognition features of individual modules need to be deciphered, so that their placement in hybrid PKSs can be restricted to catalytically productive contexts. Second, the mechanistic basis for transferring intermediates between adjacent modules must be understood, so that intermodular chain transfer can efficiently occur between heterologous modules. This report provides new insights into the relative importance of both of these issues and their interrelationships in the context of a multimodular PKS.

The tolerance and specificity of individual modules of DEBS have been indirectly investigated using a variety of genetic, biochemical, and chemical approaches.[25] Recently, it has been possible to express and reconstitute individual DEBS modules as intact proteins.[23] This allowed us to directly assess the substrate specificities of four modules of DEBS (modules 2, 3, 5, and 6) using a set of *N*-acetylcysteamine (NAC)-activated diketides as potential substrates (**2a**−**d**, Figure 2A).[26] Surprisingly, not only did the substrate specificity profiles of these four individual modules turn out to be quite similar, but these profiles also did not correlate well to the structures of the natural substrates of individual modules. Separately, recent experiments have suggested that short intermodular linker sequences play an important role in the selective transfer of polyketide intermediates between modules.[23,27] Integrating these two issues,

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we considered it appropriate to reexamine the steady-state kinetic parameters of individual DEBS modules, but this time, to pay closer attention to possible protein−protein interactions that could be involved in passing a substrate from an upstream module to its downstream neighbor.

There are two modes by which a substrate can be passed from one module to the next. If the two successive modules are on the same polypeptide (such as modules 1 and 2 of DEBS), there is an intrapolypeptide chain transfer. On the other hand, if the two successive modules are on separate polypeptides (such as modules 2 and 3 of DEBS), there is an interpolypeptide chain transfer. In either case, biosynthetic intermediates undergo direct interthiol transfer between adjacent modules such that the intermediates never go into bulk solution. We refer to this property as the "physical channeling" of intermediates between modules.

Physical channeling (also commonly referred to as substrate channeling) is defined as a mechanism in a sequence of reactions in which reaction intermediate is transferred from one active site to the downstream active site without equilibrating with the bulk solution.[28] Physical channeling of intermediates can provide kinetic benefits by increasing the effective concentration of the substrate, protecting labile intermediates from unproductive reactions, and precluding entrance of intermediates into competing enzymatic pathways. Furthermore, substrate channeling between two enzymes can help overcome product inhibition of the upstream enzyme by funneling the intermediate out of the upstream binding pocket and into the downstream binding pocket more efficiently.

While physical channeling is a necessary outcome of fundamental polyketide biosynthetic mechanisms,[17,29] the kinetic advantage, if any, of channeling intermediates between modules has not yet been resolved. To elucidate the issue of "kinetic channeling" (which is defined as physical channeling that results in a kinetic advantage—as measured by k_{cat}—over a diffusive loading mechanism in which the intermediate equilibrates in the bulk phase after release from the upstream active site and before loading in the downstream active site) in modular PKSs, two new assay systems—one to probe intrapolypeptide transfers and one to probe interpolypeptide transfers—were devised that would more accurately mimic the transfer of a substrate from the acyl carrier protein (ACP) of one module to the ketosynthase (KS) of the next. In the first assay system, the loading didomain and module 1 of DEBS generated in situ the natural diketide intermediate ((2*S*,3*R*)-2-methyl-3-hydroxy-pentanoyl-S-ACP$_1$), which could then be transferred to alternative downstream modules in a bimodular PKS context (Figure 2B). By comparing the kinetic parameters of these hybrid bimodular systems to those for elongation of the same diketide that has been supplied exogenously to the isolated downstream module (Figure 2A), the kinetic benefit of channeling intermediates between covalently linked modules could be evaluated. A second assay system was developed using a chemoenzymatic method, through which alternative diketides were covalently attached to the phosphopantetheine arms of an individually expressed donor ACP domain (Figure 2C). Here, the entire diketide-S-ACP adduct (**4a**−**d**) is a formal substrate for a recipient module, therefore allowing investigation of interpolypeptide channeling. (The linker sequence at the C-terminal end of the ACP as previously described[27] was included in this construct.) By attaching different diketides to the same ACP, the steady-state kinetic parameters for diketide elongation by individual modules (each with a TE domain fused to its C terminus to facilitate turnover) could be measured. Both assay systems were used to compare the properties of modules 2, 5, and 6 of DEBS, three modules that perform the same chemistry with identical stereocontrol, albeit on very different substrates (Figure 1).

Results

Nomenclature. The nomenclature used in this report for proteins containing linker regions is similar to that adopted in a previous publication.[27] Specifically, the module of origin of the linker is placed in parentheses either before or after the name of the domain or module to which it is attached, depending on whether it is an N- or a C-terminal linker, respectively. For example, a fusion protein comprising the ACP domain from module 2 of DEBS and the C-terminal linker of module 4 is referred to as ACP2(4); likewise, a protein comprising the N-terminal linker of module 5 fused to module 6 is referred to as (5)M6.

Construction and Expression of Bimodular Enzymes. Analogous to DEBS1+TE described earlier,[6,7] M1+M5+TE (module 1 + module 5 + TE) and M1+M6+TE are heterologous fusions of DEBS module 1 with DEBS modules 5 and 6, respectively. The natural linker between modules 1 and 2 in the wild-type DEBS1 protein was preserved in each case. In addition, the DEBS thioesterase (TE) domain was fused to the C termini of each downstream module to facilitate turnover by catalyzing the release of the triketide product. These two proteins were expressed as C-terminally His_6-tagged proteins and purified on a hydrophobic butyl sepharose column followed by a Resource Q ion-exchange chromatography to yield approximately 0.2 mg/L culture of purified M1+M5+TE and 1 mg/L culture of purified M1+M6+TE.

Kinetic Analysis of Bimodular Constructs. In earlier studies on the kinetic properties of individual modules,[26] substrates were diffusively presented to the KS domain of each module as free *N*-acetylcysteamine (NAC) thioesters. This can be contrasted with the natural mode of chain transfer in a multimodular system, where acyl chains arrive at the KS domain via direct transfer from an upstream ACP domain (Figure 1). To explore whether the latter mode of substrate incorporation can have kinetic benefits over the former, the rates of triketide lactone **3a** synthesis by M1+M5+TE and M1+M6+TE were measured in the presence of saturating concentrations of propionyl-CoA, methylmalonyl-CoA, and NADPH. The k_{cat} values for these two hybrid PKSs were determined to be 3.1 ± 0.1 and 4.1 ± 0.4 min^{-1}, respectively (Figure 4). These parameters compare well with the maximal rate of 4.8 min^{-1} for DEBS1+TE,[30] In contrast, we have shown earlier that whereas module 2+TE and module 6+TE turn **2a** over with comparable rate constants (k_{cat} = 4.6 and 17 min^{-1}, respectively), module 5+TE is a significantly weaker catalyst for the same reaction (k_{cat} = 0.25 min^{-1}). Addition of exogenous **2a** to the reaction catalyzed by the bimodular proteins had no effect on their overall catalytic rates. The implications of these results to intramodular substrate channeling will be evaluated in the discussion section.

Construction and Expression of Individual ACPs. ACP4-(4) includes the entire DEBS ACP4 catalytic domain with its natural C-terminal linker. (The ACP linker is defined as the residues between the ACP consensus sequence and the C terminus of the polypeptide.[27]) This gene was expressed as a 20.5 kDa N-terminally His_6-tagged protein to preserve the

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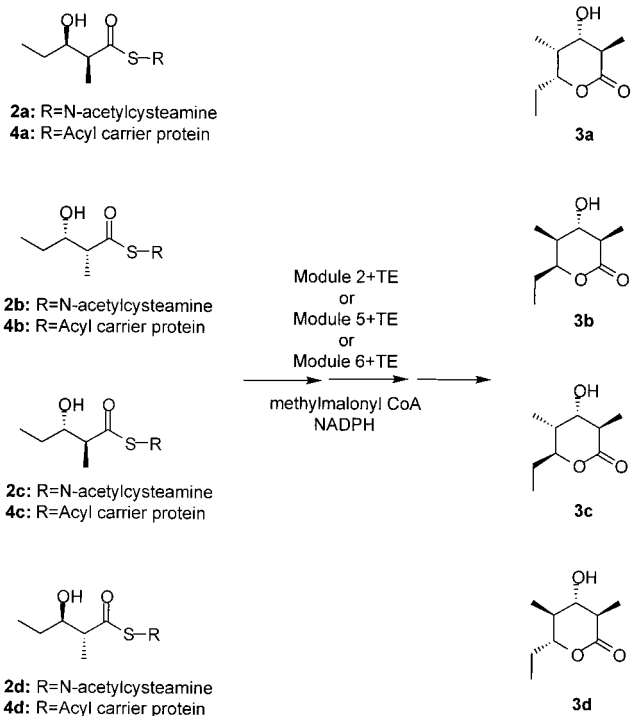

Figure 3. The four diketides and their corresponding, putative enzymatic products. See also caption to Figure 2.

natural sequence of the C-terminal linker. ACP4(4) was purified by affinity chromatography on a nickel column followed by a hydrophobic phenyl sepharose column to yield approximately 10−15 mg/L culture of purified apoprotein.

Chemoenzymatic Synthesis of Acyl-ACPs. Preparations of the CoA thioesters of the natural diketide substrate of module 2, its enantiomer, its C-3 epimer, and its C-2 epimer (Figure 3) were carried out as described in the Materials and Methods section. Phosphopantetheinylation of apo-ACP4(4) was catalyzed by Sfp[31,32] (Figure 2C, first reaction) to generate acyl-ACP4(4) adducts **4a**−**d**. Purification of these acyl-ACPs, as described in the Materials and Methods section, led to >95% pure materials as judged from SDS-PAGE. Complete phosphopantetheinlyation was verified by MALDI-MS or ESI-MS.

Qualitative Assays of Diketide Incorporation by Acyl-ACPs. The acyl-ACP4(4) adducts **4a**−**d** were incubated individually with (5)M2+TE, (5)M5+TE, and (5)M6+TE in the presence of saturating concentrations of ^{14}C-methylmalonyl CoA extender unit and NADPH. For a given acyl-ACP, the products from modules 2+TE, 5+TE, and 6+TE were expected to be identical (Figure 3), since the modules catalyze the same set of reactions with identical stereocontrol (albeit normally on very different natural substrates). Both **4a** and **4b** were accepted and extended by all three modules. Likewise, the corresponding NAC-thioesters **2a** and **2b** have been shown to be substrates for the three modules.[26] Remarkably, however, **4c** and **4d** were also substrates for the three modules, even though no turnover of the corresponding NAC thioesters **2c** and **2d** was detected in the case of any module.[26] (It should be noted that elongation of **4c** and **4d** by modules 5+TE and 6+TE yielded minor quantities of unreduced triketide lactones, indicating less efficient β-ketoreductase activity on these two anti-diketide substrates than on the two syn-diketide substrates.) Consistent with previous linker studies,[23,27] while all four acyl-ACP adducts were observed to be substrates for (5)M3+TE, no product formation was observed from the incubation of any of the acyl-ACP adducts with (3)M3+TE, even though **2a** and **2b** have previously been shown to be readily incorporated and elongated when presented to either module 3+TE derivative.[26,27] Thus, matched linker pairs appear to be capable of enhancing the efficiency with which otherwise poor substrates can be channeled between modules. Conversely, mismatched linkers can present a major barrier to the channeling of otherwise acceptable substrates between modules. Control studies performed with **2a** and **5a** showed that the two compounds are approximately equivalent substrates for the same modules (data not shown).

From the amount of product detected in these ACP-mediated reactions, the efficiency of the PKS-catalyzed reaction could be estimated. Under typical assay conditions (20 μM **4** and 1 μM (5)M5+TE), 70% of the acyl-ACP was converted into triketide lactone in 1 h. Two conclusions can be drawn from this result. First, acyl-ACPs are significantly superior substrates to acyl-NAC thioesters. (Typically, millimolar concentrations of the NAC thioester must be used to detect comparable amounts of product under otherwise similar assay conditions.) Second, the assay system described in Figure 3 allows for monitoring multiple turnovers of the enzyme. Indeed, as described below, in all cases the maximum rates of consumption of the acyl-ACP substrates were comparable to or higher than the maximum rates of consumption of their NAC thioester counterparts (see below). Therefore, the association of the donor ACP and the acceptor module must be transient, and the dissociation rate constant of the ACP from the module must be significantly faster than the slowest step in the module-catalyzed elongation sequence.

Kinetic Analysis of Incorporation of Diketides from Acyl-ACPs. The k_{cat}/K_M values for the reactions of **4a** and **4b** with (5)M2+TE, (5)M5+TE, and (5)M6+TE are shown in Figure 5. Since full saturation curves could not be obtained for the ACP-bound substrates for technical reasons, the k_{cat}/K_M value for **4b** was derived by competitive assay against **2a** (whose absolute k_{cat}/K_M value was derived from the initial slope of the v versus [S] saturation curve). Likewise, the k_{cat}/K_M value for **4a** was derived by competitive assay against **4b**. Several observations are noteworthy regarding the data summarized in Figure 5. First, **2a** and **4a** are significantly better substrates than **2b** and **4b** for each module. In addition, the improvement in specificity for an acyl-ACP adduct over its NAC thioester counterpart is particularly pronounced in cases where the NAC thioesters are exceptionally poor (e.g., module 2+TE-catalyzed elongation of **4b** versus **2b**, module 5+TE-catalyzed elongation of **4a** versus **2a**, and especially module 5+TE-catalyzed elongation of **4b** versus **2b**). The implications of these observations are elaborated in the Discussion section.

To quantify the kinetic advantage of channeling in the above assay system, the k_{cat} values for the reactions of **4a**−**d** with modules 2+TE, 5+TE, and 6+TE were measured (Figure 6). Kinetic measurements were performed at substrate concentrations between 40 and 90 μM of each acyl-ACP substrate. For reactions that yielded measurable quantities of unreduced triketide lactone products (i.e., elongation of **4c** and **4d** by modules 5+TE and 6+TE), both reduced and unreduced products were combined and included for calculations of the kinetics parameters. Except for the reaction of **4a** with module 2+TE, none of the substrates saturate the enzymes in this concentration range, and thus, the k_{cat} values are reported as lower bounds. Comparison of the k_{cat} values of the acyl-ACP

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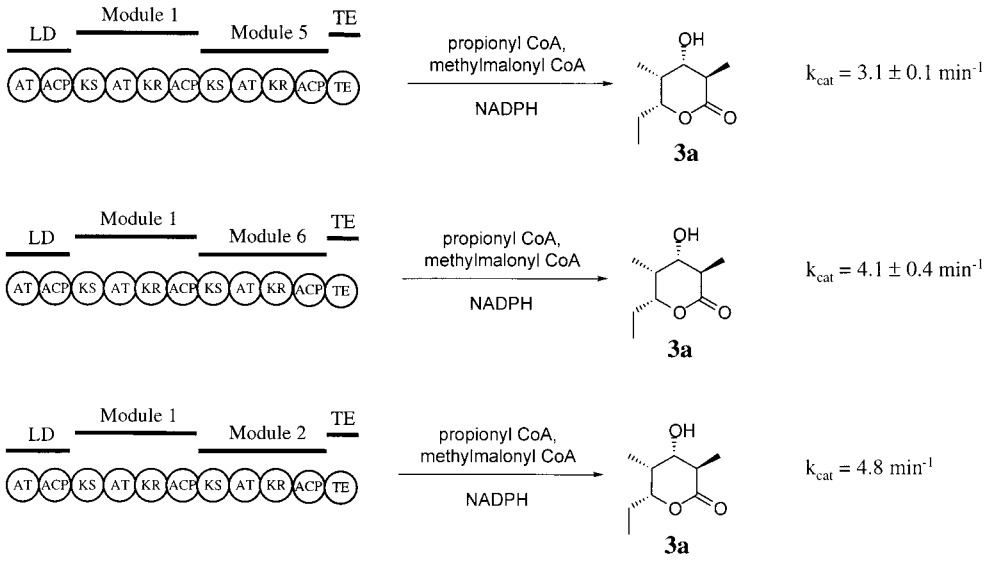


Figure 4. Reaction schemes of the three bimodular DEBS derivatives—M1+M5+TE (module 1 + module 5 + TE), M1+M6+TE, and M1+M2+TE (DEBS1+TE)—and their corresponding k_{cat} values.

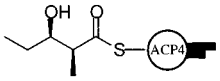
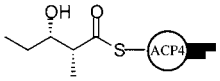
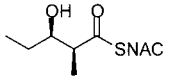
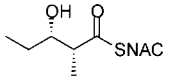
Substrate		(5)Module 2 + TE KS AT KR ACP TE	(5)Module 5 + TE KS AT KR ACP TE	(5)Module 6 + TE KS AT KR ACP TE
4a		2900 ± 500	290 ± 50	340 ± 60
4b		18 ± 1	3.9 ± 0.7	85 ± 15
2a		0.75 ± 0.01	0.016 ± 0.002	1.1 ± 0.1
2b		0.0076 ± 0.0006	0.0011 ± 0.0001	0.058 ± 0.006

Figure 5. Comparison of the k_{cat}/K_M values (min^{-1} mM^{-1}) of the two syn-diketides when presented as acyl-ACP substrates (**4a** and **4b**) vs when presented as NAC-thioesters (**2a** and **2b**). The k_{cat}/K_M values for the NAC-thioesters were reported earlier,[26] and are included by way of reference.

forms of the four diastereomeric diketides with those from the corresponding reactions involving NAC thioesters substrates (which are also shown in Figure 6)[26] affords two interesting observations. First, in those cases where the NAC thioester is a reasonably good substrate (e.g., module 2+TE- or module 6+TE-catalyzed elongation of **2a** and **4a**), the maximal reaction rates are comparable regardless of whether the acyl chain is bound to NAC or an ACP with a matched linker. In contrast, where the NAC thioester is an inferior substrate (e.g., module 2+TE-catalyzed elongation of **2b** and **4b**, M5+TE-catalyzed elongation of **2a** and **4a**, module 5+TE-catalyzed elongation of **2b** and **4b**, and elongation of **4c** and **4d** by any of the modules), tethering the same acyl chain to an ACP with a matched linker can result in significant improvements in k_{cat}. Second, the maximal rate of turnover of **2a** and **4a** is significantly greater than that of **2b** and **4b** for all tested modules. Again, the implications of these findings are discussed below.

Investigation of the Reversibility of the Donor ACP to Acceptor KS Transfer Reaction. Ordinarily, the flow of intermediates in a metabolically active PKS is vectorial. A possible mechanism for such directionality could be that, once an acceptor KS is acylated with the incoming chain, conformational changes in the module prevent the pantetheine arm of the donor ACP from accessing the active site again. To test whether this may be the case, holo-ACP4(4) was incubated with ^{14}C-**2a** in the presence and absence of (5)M2+TE (Figure 7). The ACP was radiolabeled only in the reaction containing (5)M2+TE. (As expected, (5)M2+TE was also labeled.) When methylmalonyl CoA extender units and NADPH were added to induce catalytic activity of the module, (5)M2+TE dependent labeling of ACP4(4) was also observed, but the degree of labeling was considerably reduced. Thus, there does not appear to be an absolute barrier to the back-transfer of an acyl chain from a KS to the ACP of the preceding module.

Discussion

We have previously investigated the substrate specificity of individual modules of DEBS using diketide substrates activated as *N*-acetylcysteamine (NAC) thioesters (Figure 2A).[26] The diketides included in the previous study were the four diastereomeric forms of the natural substrate for module 2 (Figure 3).

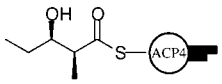
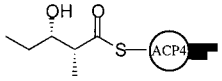
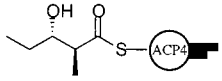
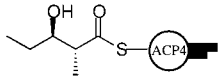
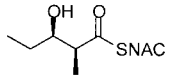
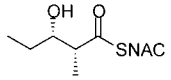
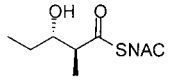
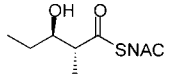
Substrate	(5)Module 2 + TE (KS AT KR ACP TE)	(5)Module 5 + TE (KS AT KR ACP TE)	(5)Module 6 + TE (KS AT KR ACP TE)
4a 	6.7 ± 0.2	> 9.3 ± 1.4	> 10 ± 1
4b 	> 0.97 ± 0.02	> 0.48 ± 0.02	> 3.4 ± 0.4
4c 	> 1.0 ± 0.1	> 1.4 ± 0.1	> 2.1 ± 0.2
4d 	> 0.29 ± 0.03	> 0.20 ± 0.01	> 1.9 ± 0.1
2a 	> 4.6 ± 0.6	0.24 ± 0.01	17 ± 2.9
2b 	0.25 ± 0.02	0.017 ± 0.001	2.4 ± 0.2
2c 	N. D.	N. D.	N. D.
2d 	N. D.	N. D.	N. D.

Figure 6. Comparison of the k_{cat} values (min^{-1}) of the four diketides when presented as acyl-ACP substrates (**4a**−**d**) vs when presented as NAC-thioesters (**2a**−**d**). "N.D." denotes that the product was not detected.

These substrates were assayed against DEBS modules 2, 3, 5, and 6—each with a TE domain fused to the C terminus to facilitate turnover—and steady-state kinetic parameters were determined for each substrate−enzyme combination. The substrate specificity profiles (as reflected in the k_{cat}/K_M values) for the four enzymes were found to be remarkably similar in that all of the modules preferred **2a** over **2b**, and, within detection limits, neither of the two anti-diketides **2c** or **2d** was observed to be a substrate for any of the modules.

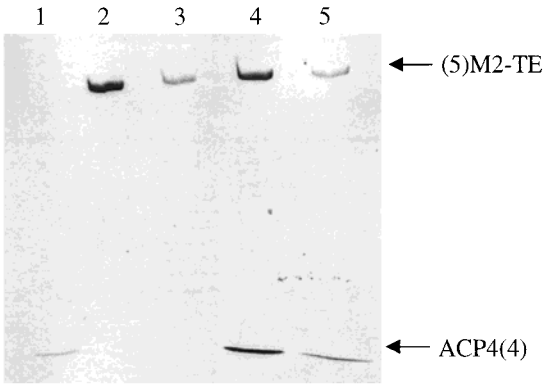
The preference of **2a** over its enantiomer **2b** for all modules was especially intriguing in light of the fact that the natural substrates for modules 3 and 6 share more structural similarities to **2b** than to **2a**. One explanation for this discrepancy was that the NAC thioester-based assay system (Figure 2A) may not entirely represent the mechanism of acylation of a multimodular system. While NAC thioesters substrates must be loaded diffusively onto the KS of a module (Figure 2A), polyketide intermediates are channeled from the ACP of one module to the KS of the downstream module via covalent transfer. Substrate channeling in a multimodular system can occur either between two modules on the same polypeptide (e.g., between modules 1 and 2; Figure 2B), or between two modules on separate polypeptides (e.g., between modules 2 and 3; Figure 2C). Further evidence that protein−protein interactions may influence the substrate specificities of individual modules emerged from previous experiments suggesting, that inter-polypeptide linkers—defined as the highly variable regions outside the consensus sequences of the modules—are involved in mediating selective intermodular chain transfer.[23,27] To investigate the balance of protein−protein interactions and enzyme−substrate interactions in controlling polyketide chain elongation, two assay systems that take intermodular interactions into account were used in this study.

Kinetic Channeling in Intrapolypeptide Chain Transfer. In the first system (Figure 2B), the effect of substrate channeling between two modules within the same polypeptide was investigated. More specifically, in the context of the bimodular constructs M1+M2+TE (DEBS1+TE), M1+M5+TE, and M1+M6+TE, DEBS modules 2, 5, and 6 were examined for their abilities to accept and elongate the natural diketide intermediate that was passed from a covalently attached module 1. The turnover number of M1+M5+TE was comparable to that of M1+M6+TE or the "wild-type" M1+M2+TE. In contrast, when primed diffusively by **2a**, the maximum catalytic rate of module 5+TE is significantly reduced compared to that of module 2+TE or module 6+TE. This disparity indicates that covalent connection of modules can have a beneficial kinetic effect and hinted that, in addition to physical channeling of intermediates, multimodular PKSs are also capable of kinetic channeling of intermediates. However, since the only incoming substrate that could be tested using this assay system was the natural diketide, a better assay system was needed to explore the role of kinetic channeling more generally.

Kinetic Channeling in Interpolypeptide Chain Transfer. The minimal donor protein requirement for substrate channeling to an acceptor module was postulated to be an ACP domain with an appropriate C-terminal linker. Therefore, we constructed, expressed, and purified the ACP4 domain and its natural C-terminal linker as an individual polypeptide. A variety of acyl groups were then covalently attached to the phosphopantetheine

A

Lane	ACP	Module	^{14}C-Label	Extender Unit
1	holo-ACP4(4)	none	* **2 a**	none
2	none	(5)M2+TE	* **2 a**	none
3	none	(5)M2+TE	* **2 a**	methylmalonyl-CoA
4	holo-ACP4(4)	(5)M2+TE	* **2 a**	none
5	holo-ACP4(4)	(5)M2+TE	* **2 a**	methylmalonyl-CoA



B

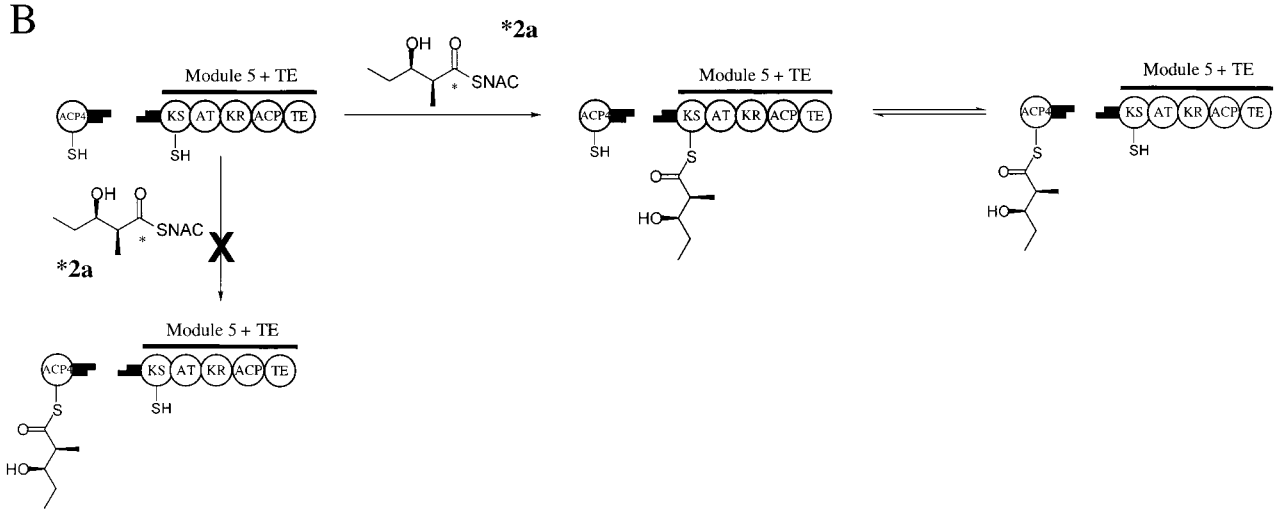


Figure 7. (A) X-ray film image of SDS-PAGE gel and associated conditions of back-transfer experiments. (B) Proposed mechanism for back transfer of an exogenously loaded diketide from the KS of a formally downstream module to an upstream ACP.

arm of holo-ACP4(4) via a chemoenzymatic procedure (Figure 2C, first reaction). The resulting acyl-ACP4(4) adducts **4a**−**d** were tested for their ability to transfer the attached diketides from ACP4(4) to the KS of an acceptor module (Figure 2C, second reaction), where they could then undergo standard chain elongation to yield a triketide lactone (Figure 2C, third reaction). The small size of the ACP4(4) protein, together with high expression levels of soluble protein in *Escherichia coli*, allowed production of reagent quantities of this protein for use as a substrate in multiple turnover assays. The requirement of both an ACP and the linker was highlighted by the fact that corresponding CoA thioesters exhibited comparable kinetic parameters and that mismatched linkers led to a dramatic reduction in turnover efficiency in the case of module 3+TE. The latter feature is consistent with the linker hypothesis developed earlier.[23,27] Although the precise K_M values for individual acyl-ACP substrates could not be measured in many cases, in all cases they were estimated to be approximately 2−3 orders of magnitude lower than the reported K_M values for the corresponding NAC thioester reactions (micromolar for acyl-ACPs versus millimolar for acyl-S-NACs), thus making acyl-ACPs excellent substrates for individual PKS modules.

Implications of the Interpolypeptide Transfer Kinetics Data. The establishment of the acyl-ACP-based assay system allowed us to address two important questions regarding the relative balance of protein−protein interactions and enzyme−substrate interactions in multimodular systems. First, is the universal preference among the three tested modules for **2a** over **2b** preserved when the same substrates are delivered as acyl-ACP adducts? And second, under saturation conditions, can kinetic channeling of these diketide substrates be observed for any module?

As seen in Figure 5, the preference for the (2*S*, 3*R*)-diastereomer over its enantiomeric (2*R*, 3*S*)-diastereomer by all modules is preserved regardless of whether the substrate is loaded by a channeling mechanism or by a diffusive mechanism. This conserved preference suggests that the catalytic steps in a given module that discriminate between different substrates remain unchanged whether modules are primed diffusively or by a channeling mechanism and that for module 2, at least, the most likely source of discrimination is the KS acylation step. Furthermore, the turnover numbers of the individual enzyme-catalyzed reactions reported in Figure 6 make a strong case for kinetic channeling in multimodular PKSs. When saturating concentrations of **2b** are co-incubated with M5+TE in the presence of methylmalonyl-CoA and NADPH, the elongation rate constant is only 0.02 min^{-1}. In contrast, when the same reaction is monitored using **4b** as a substrate, the elongation rate constant (k_{cat}) increases at least 25-fold. Similarly, a maximal rate increase of greater than 40-fold is observed in the elongation of **4a** versus **2a** by M5+TE. The effect may be even more pronounced for the two anti-diketides, whose elongation rates are below detectable limits ($<$0.01 min^{-1}) when presented as NAC thioesters **2c** and **2d**, but are quite respectable ($\sim$1 min^{-1}) when presented as acyl-ACP adducts **4c** and **4d**. Of course, if the K_M values for the two anti-diketides when presented as NAC-thioesters are significantly higher than the solubility-limited concentrations that were used in the assay,

then the apparent kinetic advantage of channeling the antidiketides could be artificially high. Even so, these overall results indicate that channeling dramatically increases the efficacy of poor substrates. In addition, the results reported here provide insight into how multimodular PKSs can be so remarkably tolerant toward protein engineering, even though individual modules are fairly specific catalysts.

The Reversibility of ACP$_n$ to KS$_{n+1}$ Transfers. Finally, the ACP-mediated strategy for diketide loading onto acceptor modules also enabled us to address the question of reversibility of the transacylation reaction between the donor ACP and the recipient KS. While co-incubation of ^{14}C-labeled **2a** with holo-ACP4(4) afforded essentially no labeling of the ACP, co-incubation of ^{14}C-labeled **2a** with holo-ACP4(4) in the presence of (5)M2+TE gave both labeled (5)M2+TE and ACP4(4) (Figure 7A). The proposed mechanism for the observed labeling is shown in Figure 7B and requires back-transfer of the acyl group from the KS to the ACP of the upstream module. Back-transfer was also observed under turnover conditions, albeit at a substantially reduced level. Thus, the observed directionality of chain transfer in the context of a multimodular PKS that is rapidly turning over appears to arise due to kinetic channeling of these intermediates rather than a ratchet mechanism that explicitly precludes back-transfer. However, given the 20-fold excess of ACP to module, the occupancy level of the diketide on the ACP is quite low. Consequently, in a PKS where two modules on separate polypeptides exist in approximately equimolar ratios, reverse transfer from a downstream module to an upstream module occurs rarely and only at steps where a significant barrier for forward chain transfer is encountered. In contrast, for intermodular chain transfer between modules within the same polypeptide, the effective molarity of the donor ACP group is significantly higher, and reversible transfer may be more significant (but without chemical consequence). The likelihood of intrapolypeptide reverse transfer may explain why the loading didomain shows relatively low selectivity for a propionyl starter unit versus an acetyl starter unit (2:1),[33] whereas DEBS1+TE discriminates strongly (32:1) between the two starter units.[34]

In summary, this report represents the first direct observation of kinetic channeling of intermediates in a modular PKS. Several dramatic examples are presented for both intrapolypeptide transfers and interpolypeptide transfers where the maximal rate constant (k_{cat}) for elongating a particular ketide substrate by a DEBS module increases 10- to >100-fold when the substrate is channeled relative to when it is diffusively presented. Linkers are shown to play an important role in kinetic channeling, although the contribution of other elements, such as the pantetheine arm or protein−protein interactions between the donor and recipient modules, cannot be excluded. In addition, our studies have also reinforced the fact that, while individual modules are tolerant of stereochemical diversity in diketides, they are at the same time fairly specific catalysts. In addition, their specificities and recognition features do not necessarily correlate with the structures of their natural substrates. Finally, we have shown that the transfer step from a donor ACP to an acceptor KS is a fundamentally reversible reaction. Structural and more detailed mechanistic studies on these remarkable multifunctional catalysts should be particularly interesting from the viewpoint of understanding the atomic basis for the phenomena described here.

Materials and Methods

Construction of Plasmids. The gene encoding ACP4(4) was amplified as an *Nde*I-*Eco*RI PCR fragment (523 bp) using the primers 5′-CCATATG**GTGGTCGACCGGCTCG**-3′ and 5′-GAATTC**CTACAGGTCCTCTCCCCC**-3′ (sequences complementary to DEBS shown in bold). The PCR product was cloned into pET28a (Novagen) to yield plasmid pNW8. Plasmid pST157 encodes a bimodular fusion between module 1 of DEBS1 and module 5 of DEBS3, with the thioesterase domain fused downstream of module 5 ("M1+M5+TE"). This fusion, which was engineered by taking advantage of the natural, conserved *Bsa*BI sites located at the start of the KS domains of modules 2 and 5, also includes the loading didomain of DEBS1. The "linker" sequence that covalently bridges the fused modules is the natural sequence between modules 1 and 2, as in DEBS1. The fusion junction between module 5 and the thioesterase domain is identical to that in plasmid pRSG46.[23] Similarly, plasmid pST92 encodes an "M1+M6+TE" bimodular fusion. Its construction, which is completely analogous to that of pST157, involves introduction of this bimodular PKS gene from pST96[23] as an *Nde*I−*Eco*RI into pET-21c (Novagen). The construction of genes encoding (5)M2+TE, (3)M3+TE, (5)M5+TE, and (5)M6+TE (pRSG64, pRSG64, pRSG46, and pRSG54, respectively) have been described previously,[23] as well as the construction of a gene encoding (5)M3+TE (pST132).[27]

Expression and Purification of Proteins. All individual modules were expressed and purified as previously described.[26] The bimodular proteins were expressed as C-terminal His_6-tagged fusion proteins, and their expression and purification schemes were identical to those previously described for the individual modules,[26] yielding 0.2 mg/L culture of purified M1+M5+TE and 1 mg/mL culture of purified M1+M6+TE. ACP4(4) was expressed by transforming pNW8 into *E. coli* BL21(DE3) cells (Novagen), which were then grown in LB at 37 °C to $OD_{600} = 0.7-0.8$. BL21(DE3)/pNW8 was induced overnight with 1 mM IPTG at 30 °C. The cells were harvested by centrifugation, washed with TE buffer, and then resuspended in disruption buffer (100 mM NaH_2PO_4 (pH 7.2), 100 mM NaCl, 1.2 mM DTT, 1.2 mM EDTA, 0.7 mM benzamidine, 1 mg/L pepstatin, 1 mg/mL leupeptin, and 15% glycerol) before lysis by French press. Following removal of the cell debris by centrifugation, the supernatant was treated with 0.1% (w/v) PEI to remove nucleic acids followed by a 55% $(NH_4)_2SO4$ precipitation. The resulting $(NH_4)_2SO_4$ pellet was resuspended in 100 mM NaH_2PO_4 (pH 7.2), 2.5 mM DTT, 1 mM EDTA, 20% glycerol (buffer A). This suspension was desalted on a PD-10 gel filtration column (Amersham Pharmacia Biotech AB) equilibrated with 10 mM imidazole in 50 mM Tris (pH 8.0), 1 M NaCl, 20% glycerol (buffer B), and the eluant was loaded at 1 mL/min onto a Flex-column (Kontes) packed with 5 mL of Ni NTA-Superflow resin (Qiagen) using a peristaltic pump. After being washed with 35 mM imidazole in buffer B for ACP4-(4), the His_6-tagged protein was eluted from the resin with 90 mM imidazole in buffer B. The appropriate fractions were concentrated, and the buffers were exchanged to buffer A + 1.5 M $(NH_4)_2SO_4$ in Centriprep 10 spin columns (Amicon). Using an Akta FLPC system (Amersham Pharmacia Biotech AB), the concentrated protein was loaded at 1 mL/min onto a XK 16/20 column packed with 30 mL of Phenyl Sepharose High Performance resin and equilibrated with the same buffer. A gradient from 750 mM $(NH_4)_2SO_4$ to 0 mM $(NH_4)_2SO_4$ in buffer A was applied which eluted the protein at 0 mM $(NH_4)_2SO_4$. The appropriate fractions were concentrated in Centriprep 10 spin columns to yield approximately 10−15 mg/L of purified protein which was flash frozen and stored at −80 °C. The mass of apo-ACP4(4) was confirmed by MALDI-MS (calculated mass: 20492, observed mass: 20507). (MW − methionine) was also observed.

Synthesis of CoA Thioester Diketides. The carboxylic acids of the diketides were synthesized as previously described.[35] They include the (*2S*,*3R*), (*2R*,*3S*), (*2R*,*3R*), and (*2S*,*3S*) diastereomers of 2-methyl-3-hydroxy-pentanoic acid. These carboxylic acids were converted to CoA thioesters **5a**−**d** under the following conditions.[36,37] Carboxylic acid

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(34) Pieper, R.; Ebert-Khosla, S.; Cane, D.; Khosla, C. *Biochemistry* **1996**, *35*, 2054−2060.

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(36) Belshaw, P. J.; Walsh, C. T.; Stachelhaus, T. *Science* **1999**, *284*, 486-489.

(3.4 mg, 26 μmol), CoASH (sodium salt, 1.1 equiv, Sigma), and PyBOP (1.5 equiv, Novabiochem) were dissolved in 0.39 mL of THF and 0.39 mL of 4% K_2CO_3 and stirred under argon for 40 min. The reaction mixture was diluted to up 5 mL with H_2O and injected onto a Beckman Ultrasphere C_{18} HPLC column (250 × 10 mm) equilibrated with 50 mM NaH_2PO_4 (pH 4.2) in 10% MeOH/H_2O. Using a 10 mL/min linear gradient over 30 min to 50 mM NaH_2PO_4 (pH 4.2) in 80% MeOH/H_2O, the CoA thioesters eluted at 55% MeOH. After removal of the MeOH on a rotavap, the product was desalted by reinjection on the same column equilibrated with 10% MeOH/H_2O followed by elution with 90% MeOH. The product was lyophilized and verified by MALDI-MS (theoretical mass: 881.742; observed mass: 882.191) and ^{1}H NMR (500 MHz) in H_2O. **5a**: 0.71 (s, 3H), 0.85 (s, 3H), 0.86 (t, 3H), 1.08 (d, 3H), 1.48 (m, 2H), 2.38 (t, 2H), 2.76 (m, 1H), 2.96 (t, 2H), 3.28 (t, 2H), 3.41 (t, 2H), 3.52 (dd, 1H), 3.73 (td, 1H), 3.79 (dd, 1H), 3.98 (s, 1H), 4.20 (t, 2H), 4.55 (t, 1H), 4.79 (m, 2H), 6.13 (d, 1H), 8.21 (s, 1H), 8.51 (s, 1H). **5b**: 0.72 (s, 3H), 0.86 (s, 3H), 0.89 (t, 3H), 1.11 (d, 3H), 1.44 (m, 2H), 2.40 (t, 2H), 2.79 (m, 1H), 2.97 (t, 2H), 3.30 (t, 2H), 3.43 (t, 2H), 3.52 (dd, 1H), 3.75 (td, 1H), 3.80 (dd, 1H), 3.99 (s, 1H), 4.21 (m, 2H), 4.56 (m, 1H), 4.75 (m, 1H), 4.80 (m, 1H), 6.15 (d, 1H), 8.24 (s, 1H), 8.54 (s, 1H). **5c**: 0.66 (s, 3H), 0.78 (s, 3H), 0.78 (t, 3H), 0.97 (d, 3H), 1.26 (m, 1H), 1.48 (m, 1H), 2.31 (t, 2H), 2.71 (m, 1H), 2.89 (m, 2H), 3.22 (m, 2H), 3.34 (m, 2H), 3.45 (dd, 1H), 3.59 (dt, 1H), 3.72 (dd, 1H), 3.90 (s, 1H), 4.14 (m, 2H), 4.49 (m, 1H), 4.73 (m, 1H), 4.84 (m, 1H), 6.09 (d, 1H), 8.25 (s, 1H), 8.50 (s, 1H). **5d**: 0.66 (s, 3H), 0.78 (s, 3H), 0.78 (t, 3H), 0.97 (d, 3H), 1.27 (m, 1H), 1.48 (m, 1H), 2.31 (t, 2H), 2.71 (m, 1H), 2.89 (m, 2H), 3.22 (m, 2H), 3.34 (t, 2H), 3.46 (dd, 1H), 3.59 (m, 1H), 3.73 (dd, 1H), 3.90 (s, 1H), 4.14 (m, 2H), 4.49 (m, 1H), 4.75 (m, 1H), 4.82 (m, 1H), 6.09 (d, 1H), 8.25 (s, 1H), 8.50 (s, 1H). Concentrations of solutions of CoA thioesters were determined by A_{260} measurement and calibration against known CoA concentration standards. Yield: 9.6 μmol (37%).

Formation of Holo-ACP and Acyl-ACP from Apo-ACP. The phosphopantetheinylation reactions were catalyzed by the Sfp phosphopantetheine transferase[31,32] under the following conditions: 150 μM apo ACP, 4 equiv CoASH (lithium salt, Sigma) or acyl-CoA **5a**−**d**, 0.2 equiv Sfp in 100 mM NaH_2PO_4 (pH 6.6), 10 mM $MgCl_2$, 2.5 mM DTT, 20% glycerol at 37 °C for 20 min. Excess small molecules and Sfp were removed from the phosphopantetheinlyated ACPs by applying the reaction mixture with an Akta FPLC system to a 6 mL Resource Q column (Amersham Pharmacia Biotech AB) and eluting with a linear gradient from 0 mM NaCl to 500 mM NaCl in buffer A. The desired proteins eluted at 220 mM NaCl and were concentrated using Centriprep 10 spin columns. Protein concentrations were determined using a modified Lowry assay (Sigma), and the masses were confirmed by MALDI-MS or $^+$ESI-MS (**4a**: observed mass = 20945, calculated mass = 20944; **4b**: observed mass = 20964; **4c**: observed mass = 21056; **4d**: observed mass = 20992).

Qualitative Substrate Incorporation Assays. The reaction buffer for the diketide incorporation assays contained 400 mM NaH_2PO_4 (pH 7.2), 2.5 mM DTT, 1 mM ETDA, 20% glyercol (reaction buffer C). 1 μM module, 20 μM acyl-ACP, 500 μM DL-[2-^{14}C]-methylmalonyl CoA (ARC), and 4 mM NADPH (Sigma) were incubated in 20 μL of the reaction buffer at 30 °C for 1.5 h. The reactions were either spotted directly onto a TLC plate (Whatman 250 μM silica gel, UV_{254}), or first extracted with EtOAc followed by spotting of the organic extracts on a TLC plate. The TLC plates were resolved using 60% EtOAc in hexanes, and the radioactive products were visualized on a Packard InstantImager.

Verification of Reaction Products. Triketide lactone products **3a** and **3b** derived from **2a** (or **4a**) and **2b** (or **4b**), respectively, have been previously verified.[26] To verify the triketide lactone products **3c** and **3d**, reaction extracts were purified by preparative TLC. The ethyl acetate extracts of the spots corresponding to the triketide lactones were concentrated and then derivatized to TMS ethers by incubation with 50 μL of *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (Aldrich) for 30 min at room temperature.[38] Injection of the sample onto a GC−MS yielded fragmentation peaks at molecular weights 73 and 171, corresponding to cleavage between the oxygen and silicon atoms, as expected. Mass spectral confirmation data of the β-ketolactone equivalents of **3c** and **3d** were obtained sans derivatization and by ESI-MS. The elution pattern of the triketide lactones from a chiral HPLC column is described below.

Determination of k_{cat} Values. The assays for kinetic measurements were performed in reaction buffer C and with the same concentrations of NADPH and ^{14}C-methylmalonyl CoA as for the qualitative assays. Saturating concentrations of propionyl-CoA were added to and the ACP substrates were excluded from the bimodular reactions. To quench the reactions, 20 μL reaction aliquots were mixed with 80 μL of 12.5% SDS. k_{cat} values for the acyl-ACP substrates were determined by measuring steady-state saturating rates at multiple substrate concentrations (varying from 40 to 90 μM **4**). For reactions that did not saturate by 90 μM of substrate, the k_{cat} values are reported as lower limits. Workup and visualization of the reaction products were identical to those for the qualitative assays.

Determination of k_{cat}/K_M values. The assays for determination of $(k_{cat}/K_M)_{rel}$ were performed with two competing substrates in the same reaction under the same conditions as described above for the qualitative assays, except the reaction volumes were doubled to 40 μL. The data were fit into the equation where S_A and S_B are the two competing substrates and P_A and P_B are the corresponding products derived from S_A and S_B, respectively. The unknown, absolute k_{cat}/K_M values could then be obtained from known, absolute k_{cat}/K_M data that had been derived directly from the initial slopes of v versus [S] plots.[26] Each reaction was done in duplicate at two different ratios of substrate concentrations. The reactions were quenched with 120 μL of 12.5% SDS, and the products were extracted with 2 × 300 μL of EtOAc. The organic extracts were purged of highly polar compounds as well as particulates by flash chromatography through 50 μL of silica gel in a 1-mL polypropylene pipet attached to a 3-mm, 0.22-μm nylon syringe filter (Osmonics, Inc.), eluting with 1.5 mL of EtOAc. Following removal of the organic solvents, the residual extracts were resuspended in 20 μL of hexane and loaded onto a 250 × 4.6 mm Chiralpak AS column with the corresponding guard column (Daicel Chemical Industries) that had been equilibrated with 5% EtOH (Reagent Alcohol, Fischer) in hexane. With a flow rate of 0.8 mL/min, the products were separated using a 20 min gradient (starting at 2 min) from 5 to 15% EtOH in hexane. The reduced triketide lactone products **3a**−**d** eluted at 20.0, 17.0, 21.5, and 18.5 min, respectively. The unreduced triketide lactone products, derived from **4c** and **4d**, eluted at 21.0 and 19.0 min, respectively. The appropriate fractions were collected, and the radioactive products were detected and quantified using Formula-989 liquid scintillation cocktail fluid (Packard) on a Beckman LS3801 liquid scintillation counter.

Labeling of Holo-ACP4(4) with ^{14}C-2a Mediated by (5)M2+TE. Holo-ACP4(4) (20 μM) was incubated with 1 mM [1-^{14}C]-labeled **2a** (custom synthesized by Amersham Pharmacia, specific activity 55 mCi/mmol) and 1 μM (5)M2+TE in reaction buffer C for 10 min at 30 °C. The protein was precipitated with 75% acetone/H_2O for 5 min at −80 °C. After washing the pellet with 6.25% (w/v) TCA to remove excess salts followed by 500 μL of 75% acetone/H_2O to remove residual, unbound ^{14}C-**2a**, the precipitated protein was resuspended in 8 μL of buffer A and 4 μL of SDS sample buffer, and resolved on a 4−20% SDS−PAGE gradient gel (Bio-Rad). The proteins were visualized with Coomassie blue stain and dried, and the radioactivity was detected either on a Packard InstantImager or by exposing the gel to X-ray film.

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