In Vivo and In Vitro Analysis of the Hedamycin Polyketide Synthase

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SUMMARY

Hedamycin is an antitumor polyketide antibiotic with unusual biosynthetic features. Earlier sequence analysis of the hedamycin biosynthetic gene cluster implied a role for type I and type II polyketide synthases (PKSs). We demonstrate that the hedamycin minimal PKS can synthesize a dodecaketide backbone. The ketosynthase (KS) subunit of this PKS has specificity for both type I and type II acyl carrier proteins (ACPs) with which it collaborates during chain initiation and chain elongation, respectively. The KS receives a C_6 primer unit from the terminal ACP domain of HedU (a type I PKS protein) directly and subsequently interacts with the ACP domain of HedE (a type II PKS protein) during the process of chain elongation. HedE is a bifunctional protein with both ACP and aromatase activity. Its aromatase domain can modulate the chain length specificity of the minimal PKS. Chain length can also be influenced by HedA, the C-9 ketoreductase. While co-expression of the hedamycin minimal PKS and a chain-initiation module from the R1128 PKS yields an isobutyryl-primed decaketide, the orthologous PKS subunits from the hedamycin gene cluster itself are unable to prime the minimal PKS with a nonacetyl starter unit. Our findings provide new insights into the mechanism of chain initiation and elongation by type II PKSs.

INTRODUCTION

The actinomycetes produce a variety of structurally complex aromatic antibiotics that use type II polyketide synthases (PKSs), a family of mechanistically related multifunctional enzymes (O'Hagan, 1991; Ridley and Khosla, 2009). Every type II PKS includes a core module of subunits called the "minimal PKS," which consists of the ketosynthase-chain length factor (KS-CLF) heterodimer, an acyl carrier protein (ACP), and a malonyl-CoA:ACP transacylase (MAT) that is typically shared by multiple PKSs and fatty acid synthases in the bacterium (although the MAT is required for minimal PKS activity, it is not

encoded within the PKS gene cluster) (Carreras et al., 1996; Summers et al., 1995). The minimal PKS synthesizes a highly reactive poly-β-ketothioester intermediate, which is subsequently acted upon by downstream enzymes to yield a polycyclic natural product (Das and Khosla, 2009; Hertweck, 2009; Shen, 2000). In many cases, (e.g., actinorhodin (*act*) (Carreras and Khosla, 1998) and tetracenomycin (*tcm*) (Bao et al., 1998) (Figure 1)), biosynthesis of this poly-β-ketothioester intermediate is primed by an acetyl unit (derived from the decarboxylation of a malonyl-ACP thioester) and elongated by a defined number of malonyl units (also supplied as malonyl-ACP substrates) through the iterative action of the KS-CLF (Dreier and Khosla, 2000; Dreier et al., 1999).

In other cases, the presence of a dedicated PKS initiation module leads to the formation of a nonacetyl-derived diketide that is then elongated by the KS-CLF (Hertweck et al., 2007; Moore and Hertweck, 2002). For example, the initiation module of the doxorubicin (*dps*) PKS (Grimm et al., 1994) synthesizes a propionyl-CoA derived β-ketopentanoyl-ACP intermediate, whereas the R1128 (*zhu*) initiation module catalyzes formation of a butanoyl-, pentanoyl-, hexanoyl-, or 4-methylpentanoyl-ACP thioester (Marti et al., 2000) (Figure 1). The polyketide backbones of yet other aromatic antibiotics, such as hedamycin (*hed*) (Bililign et al., 2004) and fredericamycin A (*fdm*) (Wendt-Pienkowski et al., 2005) (Figure 1), are initiated by even more complex mechanisms that remain to be elucidated.

Two key characteristics of type II PKSs that harbor dedicated initiation modules are: (i) orthogonal protein-protein specificity of KS and ACP partners from the initiation and the elongation modules of the bimodular PKS (Tang et al., 2003a); and (ii) an intimate connection between primer unit selection and chain-length specificity (Tang et al., 2004b, 2004c). The goal of this study was to gain a deeper understanding of the mechanisms of chain initiation and elongation of the hedamycin PKS, which apparently involves an unusual combination of a type I and a type II PKS systems (Bililign et al., 2004).

Hedamycin (**7**) (Figure 1), an aromatic polyketide with anticancer activity (Bradner et al., 1966; Joel and Goldberg, 1970), is produced by *Streptomyces griseoruber* (Schmitz et al., 1966) and consists of a planar 4*H*-anthra[1,2-b]pyran polycyclic aglycone with a bisepoxide side chain (Sequin et al., 1977). It also harbors two aminosugars attached via *C*-glycoside linkages (Sequin et al., 1977). The unusual side chain of hedamycin is important for its anticancer activity (Hansen et al., 1995; Owen et al., 2002; Pavlopoulos et al., 1999; Tu et al., 2005), and
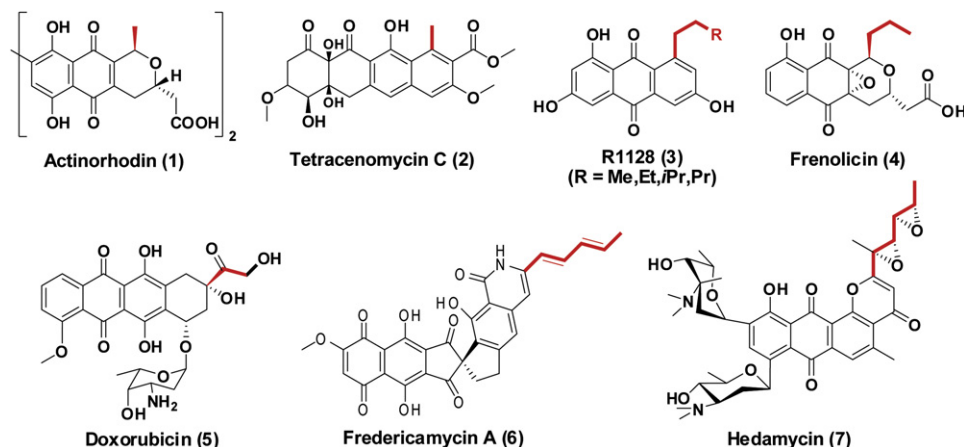
Figure 1. Structures of Acetyl and Non-acetyl Primed Aromatic Polyketides
The starter unit in each polyketide molecule is highlighted in red.

presumably corresponds to the start of the polyketide backbone (Bililign et al., 2004). Cloning, sequencing, and knockout analysis of the hedamycin gene cluster (Figure 2A) has revealed the involvement of an atypical multimodular (type I) PKS comprised of the HedT and HedU proteins in aglycone biosynthesis (Bililign et al., 2004). HedT consists of a "KS$_q$" domain responsible for the decarboxylative priming of the polyketide pathway (Bisang et al., 1999; Butler et al., 2001; Kakavas et al., 1997; Leadlay et al., 2001), an AT (acyl transferase) and an ACP domain, and has been proposed to serve as the priming module (Moore and Hertweck, 2002). HedU includes two KS domains, an AT, an ACP, a ketoreductase (KR), and a dehydratase (DH) domain. It has been proposed to catalyze two rounds of chain elongation using the acetyl primer unit supplied by HedT. Each chain elongation cycle is followed by β-ketoreduction and dehydration, leading to biosynthesis of an unsaturated 2,4-hexadienyl intermediate that is then transferred onto the minimal PKS. Disruption of *hedT* abolished the production of hedamycin, suggesting that these type I PKS proteins are involved in hedamycin biosynthesis (Bililign et al., 2004).

More typical for an aromatic polyketide gene cluster, the hedamycin gene cluster also encodes subunits that comprise the minimal PKS—a KS (HedC), a CLF (HedD), and an ACP (HedE) (Figure 2A). HedE is a bifunctional protein with an aromatase and an ACP domain. The aromatase domain of HedE is believed to catalyze the aromatization of the first six-membered ring formed during hedamycin biosynthesis. Also encoded within the gene cluster is HedA, a homolog of the *act*III gene that encodes the C-9 specific KR in actinorhodin biosynthesis (Crump et al., 1997; Korman et al., 2004). By analogy, it is thought to catalyze reduction of the C-9 carbonyl in the nascent polyketide backbone.

Intriguingly, the gene cluster also encodes a stand-alone homodimeric KS (HedS) and an acyl-ACP thioesterase (AATE, HedF), similar to the components found in the initiation modules of the doxorubicin, and R1128 PKSs (Figure 2B and Table 1). HedS, a homolog of the ketosynthase III (KSIII) found in bacterial fatty acid synthases (Rock and Cronan, 1996), is also similar to DpsC in the doxorubicin PKS (Rajgarhia et al., 2001) and ZhuH

in the R1128 synthase (Figure 2C) (Meadows and Khosla, 2001). HedF is homologous to DpsD and ZhuC, both acyl-ACP thioestearases (AATEs) that hydrolyze acetyl-primed ACPs as a proofreading mechanism (Figure 2C) (Tang et al., 2004a). Thus, the hedamycin gene cluster is the only reported bacterial PKS that incorporates elements of type I and type II PKSs. Our studies, involving experiments in vivo and in vitro, provide new insights into the mechanism and specificity of this unusual PKS.

RESULTS

In Vivo Properties of the Hedamycin Minimal PKS

A series of 12 plasmids (Table 2) containing selected combinations of PKS subunit genes were constructed, and their product profiles were analyzed in the heterologous host *Streptomyces coelicolor* CH999 (McDaniel et al., 1993a). It was unclear at first whether the hedamycin minimal PKS would behave like the R1128-minimal PKS, which does not produce polyketides in the absence of a suitable initiation module (C.K., unpublished data) or the frenolicin minimal PKS, which synthesizes polyketide products derived exclusively from malonyl-CoA in the absence of an initiation module (McDaniel et al., 1993b). To resolve this matter, two constructs were prepared harboring the *hed* minimal PKS genes with (pAD181) or without (pAD259) *hedS* and *hedF*, homologs of ZhuH (KSIII) and ZhuC (AATE) (Table 1), respectively. Both constructs also encode a C-9 specific ketoreductase (KR) (*act* KR or HedA). LC/MS and NMR analysis revealed that the same four acetyl-primed polyketides were produced by both strains as major products (**8-11**) (Figure 3). Feeding *S. coelicolor* CH999/pAD181 with precursors of alternative CoA thioesters (e.g., propionic acid, norvaline or DL-aminobutyric acid (Tang et al., 2004b)), also did not yield any nonacetyl-primed product. Detailed comparative analysis of the product profiles of the two strains failed to reveal significant differences in relative and absolute yield of products, suggesting that by themselves, neither HedS nor HedF can influence the priming mechanism of the polyketide chain (Table 1).

The dodecaketide (C-24) AD211a (**8**) (10 mg/l) was isolated from a fraction extracted with ethyl acetate/acetic acid (99:1).
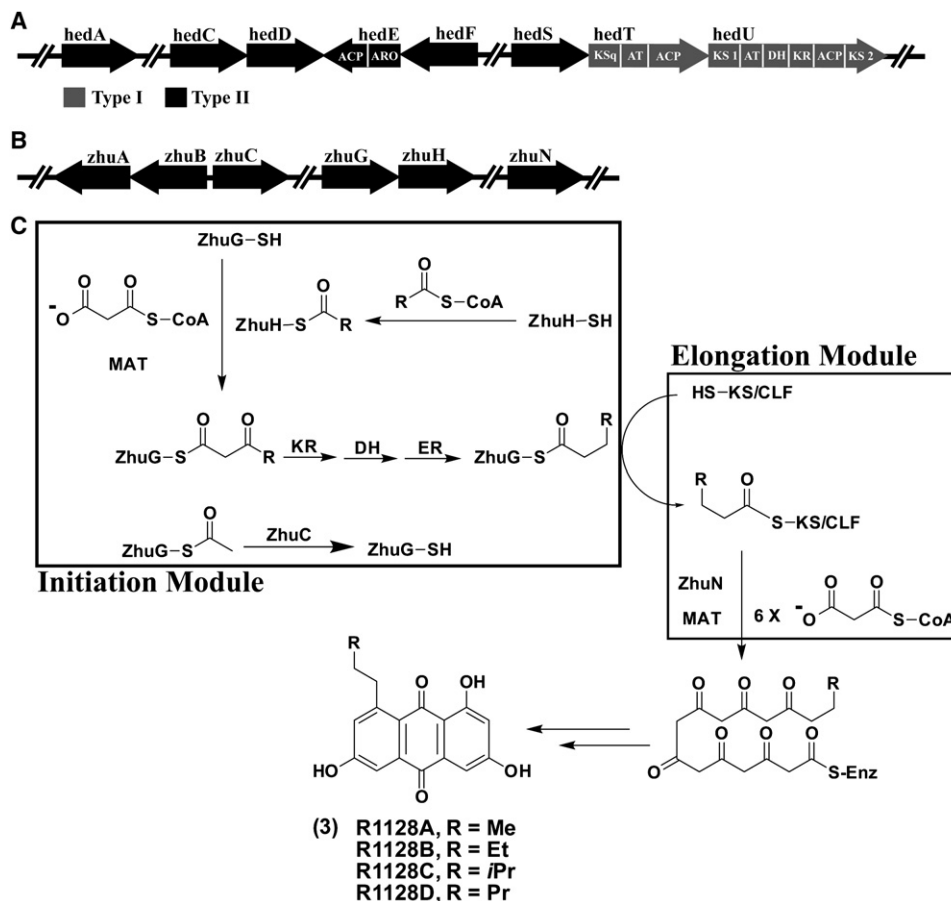
Figure 2. Comparison of the Hemadycin and R1139 Initiation Modules and Minimal PKS Genes
(A) Organization of relevant genes in the hedamycin gene cluster, which encodes both type I and type II PKS genes as shown in gray and black, respectively.
(B) Organization of relevant genes in the R1128 gene cluster. The genes are not drawn to scale. The sequences of homologous proteins in the two gene clusters were aligned; their identity/similarity is reported in Table 1.
(C) Known mechanisms for chain initiation and elongation in the R1128 PKS. The initiation and elongation modules of this PKS are shown in separate boxes.
MAT, malonyl-CoA:ACP transacylase; KR, ketoreductase; DH, Ddehydratase; ER, enoyl reductase.

LC/MS analysis revealed a mass of 435 and 433 in ESI-MS positive and ESI-MS negative modes, respectively. HR-ESI-MS confirmed that AD211a has the molecular formula $C_{24}H_{18}O_8$ (observed m/z 457.0899 $[M + Na]^+$, calcd. m/z 457.0922 for $C_{24}H_{18}O_8Na$). The structure of this previously unknown compound was unambiguously solved by ^1H and ^{13}C NMR spectroscopic analysis (Table 3), as well as HSQC, COSY and HMBC NMR experiments (see Supplemental Data available online). ^1H NMR showed the characteristic doublet-doublet peaks of H9 and doublet peaks of H8 and H10, all of which are coupled through COSY, suggesting that AD211a is C-9 reduced polyketide that has undergone a C7-C12 cyclization. Notably, the hedamycin aglycone also has an equivalent C7-C12 cyclized aromatic ring. Based on an earlier precedent, we anticipated that the ARO domain of the full-length HedE protein plays a role in AD211a biosynthesis (McDaniel et al., 1994b). To verify this activity of HedE, *S. coelicolor* CH999/pAD174 expressing the *act* KS-CLF and KR genes along with HedE was constructed and analyzed. Whereas the *act* KS-CLF, ACP and KR are known to synthesize mutactin and dehydromutactin (McDaniel et al.,

1994a), inclusion of either the *act* ARO (McDaniel et al., 1994b) or the full-length HedE (CH999/pAD174) led to the isolation of SEK34 (**12**) and SEK34b (**13**).

The structure of AD211a (**8**) also establishes that the hedamycin KS-CLF is capable of synthesizing a dodecaketide backbone, consistent with the known structure of the hedamycin aglycone. Like other KS-CLF heterodimers, it too can control the chain-length specificity by monitoring the length of the carbon-chain backbone, not the number of elongation cycles (Nicholson et al., 2003; Tang et al., 2003b). However, as discussed below, other PKS components can modulate the chain length specificity of the *hed* KS-CLF.

In addition to AD211a, two undecaketides, TW94b (**9**) and TW94c (**10**), were also isolated from *S. coelicolor* CH999/pAD181 and *S. coelicolor* CH999/pAD259 in an approximately 2:1 ratio from the fraction extracted with ethyl acetate/methanol (90:10). In an earlier report, TW94b was proposed to be converted enzymatically into the optically active (at C-14) furanone analog TW94c (Yu et al., 1998). The concomitant production of both undecaketides and dodecaketides by the *hed* KS-CLF is

Table 1. Deduced Functions and Sequence Comparison of Hedamycin and R1128 PKS Genes

Hedamycin Gene	R1128 Gene	Identity, Similarity (%)[a]	Proposed Function
hedC	zhuB	63, 17	ketosynthase (KS)
hedD	zhuA	51, 16	chain-length factor (CLF)
hedS[b]	zhuH	20, 18	ketosynthase III (KSIII)
hedF[b]	zhuC	47, 14	acyl-ACP thioesterase (AATE)
hedE			bifunctional aromatase/acyl carrier protein (ARO/ACP)
hedE ACP	zhuN	46, 26	ACP
hedE ARO/ACP			modified ARO/ACP
hedA			ketoreductase (KR)
hedT			type I enzyme
hedU			type I enzyme
hedU ACP	zhuG	25, 22	priming ACP

[a] Identity and similarity was calculated using ClustalW multiple alignment sequence server.
[b] No activity observed for these genes in vivo.

Table 2. Plasmid Constructions and the Resulting Polyketide Products

Plasmid[a]	KS-CLF	ACP(s)	Auxiliary	KR	Polyketide Products
pAD181	hed	hedE	hedF, hedS	actIII	**8, 9, 10, 11**
pAD259	hed	hedE		hedA	**8, 9, 10, 11**
pAD211	hed	hedE		actIII	**8, 9, 10, 11**
pAD258	hed	hedE(ACP)		hedA	**9, 10, 11**
pAD236	hed	hedE(ACP)		actIII	**9, 10, 11**
pAD174	act	hedE	hedF, hedS	actIII	**12, 13**
pAD212	hed	hedE			**14, 15, 16, 17, 18**
pAD175	hed	hedE	hedF, hedS		**14, 15, 16, 17, 18**
pAD185	hed	zhuN	R1128 IM		No Product
pAD188	hed	zhuN	R1128 IM	actIII	No Product
pAD180	hed	actI	actIV, actVII	actIII	No Product
pAD281	hed	hedE	R1128 IM		**14, 15, 16, 19**

[a] Each plasmid is a derivative of pRM5. The constructs were transformed into *S. coelicolor* CH999/pBOOST*. Products were analyzed by LC-MS and NMR spectroscopy. IM, initiation module.

analogous to the corresponding enzymes from spore pigment pathways (Yu et al., 1998), and suggests that the hedamycin enzyme also has relaxed chain-length specificity. Furthermore, replacing HedE with the stand-alone ACP domain from HedE (*S.coelicor* CH999/pAD236 and *S. coelicolor* CH999/pAD258) led to selective disappearance of dodecaketide **8**. Thus, the functional ARO domain of HedE is able to modulate the chain-length specificity of the KS-CLF by catalyzing the formation of an aromatic ring, as reported earlier in the cases of the *whiE* (Shen et al., 1999; Yu et al., 1998), *act*, *fren* (McDaniel et al., 1994b; Nicholson et al., 2003) and oxytetracycline (*otc*) (Petkovic et al., 1999) PKSs.

Unexpectedly, the fourth product of these two constructs was the reduced tetraketide pyrone **11** (Gruschow et al., 2007). Although its yield was lower than the other compounds (1.2 mg/l), its structure sheds new light on a fundamental property of the *act* KR that has been previously debated but never definitively established (Ma et al., 2008; McDaniel et al., 1993b). Specifically, it has been unclear whether regiospecific ketoreduction occurs on a partially elongated β-ketothioester intermediate or after formation of the full-length nascent chain. The fact that **11** harbors a hydroxyl group at C-7 suggests that the KR is able to catalyze reduction even before a full-length polyketide chain is formed. Furthermore, replacement of the *hed* KR by the *act* KR (*S. coelicolor* CH999/pAD211) did not change the product profile or the relative and absolute yields of the products, indicating that the two proteins are functionally interchangeable.

To investigate the possible influence of the *hed* KR (HedA) on chain length, a control plasmid encoding the *hed* minimal PKS without the KR was constructed and analyzed in *S. coelicolor* CH999. Removal of KR leads to an entirely different product profile. *S. coelicolor* CH999/pAD212 expressing the minimal *hed* PKS produces compounds **14, 15, 16, 17**, and **18**, whose structures were characterized by ^{1}H, ^{13}C NMR analysis, and LC/MS analysis (Supplemental Data). Compound **14** is a triketide

pyrone (Xie et al., 2006), and the tetraketide pyrone **15** (Abe et al., 2005) is the unreduced analog of **11**. Compound **16** is a pentaketide, **17** is an undecaketide, and **18** is a dodecaketide reported earlier (Yu et al., 1998) in a construct expressing the *whiE* minimal PKS. Compound **17** was the major product (8 mg/l) from *S. coelicolor* CH999/pAD212, whereas compound **18** was isolated in lower quantity (2 mg/l) and the yield of **14, 15**, and **16** was 1-2 mg/l. Consistent with the findings reported above, inclusion of *hedS* and *hedF* (*S. coelicolor* CH999/pAD175) did not lead to the production of nonacetyl-primed polyketide products (Table 2).

In Vitro Characterization of the Hedamycin Minimal PKS

To investigate the properties of the hedamycin minimal PKS *hed* KS-CLF, the stand-alone ACP domain of HedE, the bifunctional ARO/ACP derivative of HedE and *S. coelicolor* MAT were purified (Figure S1), as described in the Experimental Procedures. The identity of each of these proteins was verified by mass spectrometry (Table S5). [^{14}C]-Malonyl-CoA was incubated with various combinations of these purified proteins to identify possible covalent intermediates formed during the PKS catalytic cycle (Figure 4). When purified holo-ACP (lane 1), MAT (lane 2), or KS-CLF (lane 3) were incubated individually with [^{14}C]-malonyl-CoA, both MAT (21 Ci/mol) and ACP (15 Ci/mol) were labeled strongly. Labeling of the KS-CLF was considerably weaker (2 Ci/mol). Co-incubation of ACP (15 Ci/mol) and MAT (19 Ci/mol) with [^{14}C]-malonyl-CoA (lane 4) did not increase the label intensity on the ACP, suggesting that under non-turnover conditions, the ACP is saturated even in the absence of MAT. This is typical of certain type II ACPs that possess an intrinsic self-malonylation property (Arthur et al., 2005; Hitchman et al., 1998). When holo-ACP (11 Ci/mol) and KS-CLF (11 Ci/mol) (lane 5) were co-incubated with [^{14}C]-malonyl-CoA, both proteins were labeled. Adding MAT (19 Ci/mol) to this mixture (lane 6) further enhanced the label intensity on both ACP (28 Ci/mol) and KS-CLF (26 Ci/mol), presumably due to the increased rate of chain elongation.
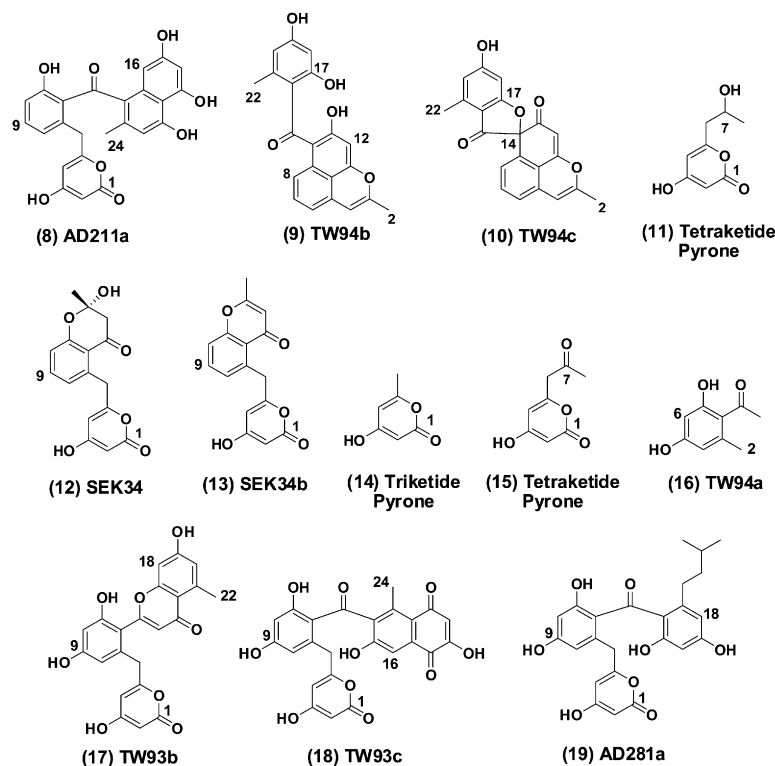
Figure 3. Structure of Polyketide Products Isolated from Different Recombinant Strains

co-expressed with the *act* ACP (*S. coelicolor* CH999/pAD180) or ZhuN (*S. coelicolor* CH999/pAD185 and *S. coelicolor* CH999/pAD188) (Table 2). None of these constructs produced detectable quantities of any polyketide. Thus it appears that the *hed* KS-CLF is able to discriminate between its cognate ACP (HedE) and orthologs from other minimal PKSs of antibiotic biosynthetic pathways, and suggests that these heterodimeric enzymes have more complex protein recognition features than had been anticipated earlier.

The absence of any influence of HedS or HedF on chain initiation or elongation led us to favor a biosynthetic model in which the type I PKS encoded by HedT and HedU was responsible for synthesizing the 2,4-hexadienyl primer unit of the hedamycin aglycone and directly priming the KS-CLF with it. However, neither HedT nor HedU could be expressed and purified to homogeneity from *E. coli* or *S. coelicolor* CH999 (data not shown). We therefore expressed and purified the ACP domain from the C-terminal end of HedU as an apo-protein (Supplemental Data). By way of comparison, two apo-ACP proteins from well-studied PKS initiation modules (ZhuG and FrenJ from R1128 and frenolicin biosynthetic pathways, respectively) were also prepared. The apo-ACPs were directly converted into the corresponding [14C]-hexanoyl-ACP by the phosphopantetheinyl transferase Sfp, as described in the Experimental Procedures, which in turn were incubated with the *hed* KS-CLF as shown in Figure 5. Due to the unavailability of [14C]-2,4-hexadienyl CoA, [14C]-hexanoyl CoA was used as a surrogate primer unit. Whereas 2 μM of [14C]-hexanoyl-ZhuG (lane 3) and [14C]-hexanoyl-HedU ACP (lane 2) were able to efficiently transfer the acyl chain onto *hed* KS-CLF, [14C]-hexanoyl-FrenJ (lane 4) could do so less efficiently. Thus, *hed* KS-CLF also appears to have some specificity for ACP subunits involved in the biosynthesis and transfer of nonacetyl primer units. The observed labeling of *hed* KS-CLF by HedU also supports the hypothesis that HedS and HedF are not required for chain transfer between the (type I) PKS initiation module and the (type II) PKS elongation module of this unusual polyketide pathway. Notably, weak labeling of the *hed* KS-CLF was observed only upon prolonged incubation with a high (100 μM) concentration of [14C]-hexanoyl-CoA (lane 1). Such a high concentration of hexanoyl-CoA is unlikely to be available in vivo and suggests that a dedicated initiation module is required to initiate hedamycin biosynthesis. Consistent with this observation and in contrast to other KS-CLF enzymes, acyl-CoA precursors such as propionic acid, DL-aminobutyric acid, and norvaline (Tang et al., 2004b) were unable to prime the biosynthesis of new polyketides, even when added to *S. coelicolor* CH999/pAD211 and *S. coelicolor* CH999/pAD212 at high concentrations ranging from 1-4 g/l (data not shown).

Surprisingly, unlike other KS-CLF heterodimers where only the KS was labeled in the presence of MAT (Carreras and Khosla, 1998; Meurer and Hutchinson, 1995), we also observed weak labeling of the *hed* CLF. Whether this species corresponds to an on-path covalent intermediate or not remains to be established. Overall, our results verify both the requirement of the KS, CLF, holo-ACP, and MAT for polyketide synthesis from malonyl-CoA, as well as the sequence of intermediates formed in the initial catalytic cycle.

The recognition between an ACP and a KS is essential for polyketide biosynthesis. Although most KS-CLF and ACP subunits from type II PKSs are interchangeable (Khosla et al., 1992, 1993; Sherman et al., 1992), there are exceptions. For example, ACPs are not exchangeable between initiation and elongation PKS modules (Tang et al., 2003a). Also for example, the ketosynthases of the initiation and elongation modules of the R1128 PKS have orthogonal specificity for their designated ACPs (i.e., ZhuG is unable to participate in chain elongation, whereas ZhuN cannot initiate chain synthesis) (Figure 2C). Also, KS-CLF and ACP subunits from antibiotic and spore pigment polyketide pathways have orthogonal specificity (Lee et al., 2005). We therefore tested the ability of several purified holo-ACP proteins from type II PKSs (Figure S1) to catalyze chain growth with the *hed* KS-CLF. When HedE was replaced with ZhuN (lane 1), FrenN (lane 2), or FdmH (lane 3), three representative ACP proteins derived from the elongation modules of the R1128, frenolicin and fredericamycin PKSs, respectively, no labeling of the *hed* KS was observed in the presence of MAT and [14C]-malonyl-CoA (Figure S2). In all cases the individual ACP proteins were labeled, confirming their intrinsic activity. The same result was verified in vivo when *hed* KS-CLF was

Table 3. ^{1}H and ^{13}C NMR Data for AD211a (8) and AD281a (19)

	AD211a (**8**)		AD281a (**19**)	
No.	^{13}C	^{1}H	^{13}C	^{1}H
1	159.2		160.7	
2	89.6	5.37 (d, J = 1.7, 1H)	88.3	5.14 (d, J = 1.7, 1H)
3	169.8		170.2	
4	108.7	6.03 (d, J = 1.7)	100.5	5.56 (d, J = 1.7, 1H)
5	154.2		159.1	
6	48.5	4.16 (s, 2H)	47.2	3.5 (s, 2H)
7	134.7		136.9	
8	109.1	6.76 (d, J = 6.8, 1H)	119.4	6.21 (d, J = 1.8, 1H)
9	128.7	7.28 (dd, J = 6.8,7.1, 1H)	160.5	
10	116.6	6.94 (d, J = 7.1, 1H)	99.6	6.08 (d, J = 1.8, 1H)
11	157		164.7	
12	119.5		108.3	
13	202.9		199.7	
14	119.5		110.1	
15	134.6		163.5	
16	105.1	6.06 (d, J = 1.6, 1H)	102.9	6.06 (d, J = 3.6, 1H)
17	163.9		160.2	
18	100.2	6.17 (d, J = 1.6, 1H)	100.3	6.15 (d, J = 3.6, 1H)
19	160.4		144.7	
20	102.8		36.8	2.18 (t, 2H)
21	155.3		30.9	1.2 (m, 2H)
22	113.2	6.63 (s,1H)	27.7	1.2 (m, 1H)
23, 23′	138		22.2	0.79 (d, J = 0.54, 6H)
24	19.9	1.95 (s, 3H)		

^{1}H and ^{13}C NMR data were recorded in DMSO-d_6 (500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts are reported in δ (ppm). Coupling constants are reported in Hz. Carbons are labeled according to their number in polyketide backbone.

To deduce the precise pathway of acyl chain transfer between the ACP domains of initiation and elongation modules, HedE ARO/ACP was added after labeling the *hed* KS-CLF with [^{14}C]-hexanoyl-ZhuG (Figure 6). No labeling of the ARO/ACP was observed in the absence of the KS-CLF (lane 1). When *hed* KS-CLF was added, chain transfer was readily observed (lane 3). No transacylation was observed when [^{14}C]-hexanoyl HedU ACP and HedE ARO/ACP were co-incubated by themselves (data not shown). Thus a model for chain growth can be derived (Figure 7) in which the *hed* KS-CLF first receives the primer unit from the ACP domain of HedU and only then engages with HedE to catalyze further chain elongation.

In Vivo Demonstration of Interaction between the Hedamycin Minimal PKS and an Initiation Module

The above data suggested that the *hed* KS-CLF could accept nonacetyl primer units only from selected initiation modules. Due to our inability to express the type I PKS proteins from the hedamycin PKS in *E. coli* or *S. coelicolor,* and guided by our in vitro data summarized above, we tested this hypothesis with the R1128 initiation module. Indeed, coexpression of the
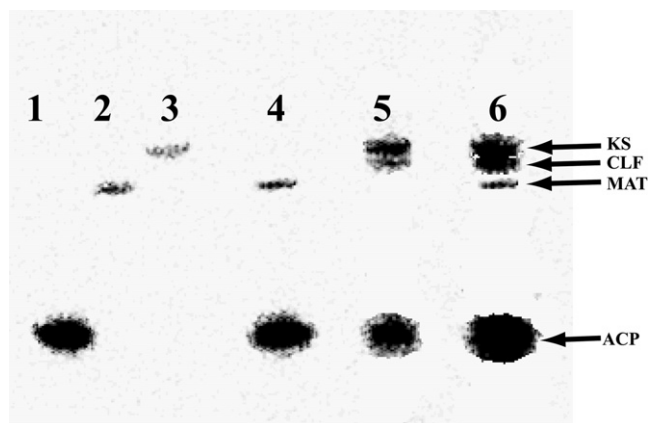

Figure 4. Autoradiogram of a 4%–12% SDS-PAGE Gel Showing Labeling of Purified PKS Proteins by [^{14}C]-malonyl-CoA
Lane 1, holo-HedE ACP only; lane 2, *fabD* MAT only; lane 3, *hed* KS-CLF only; lane 4, holo-HedE ACP + MAT; lane 5, holo-HedE ACP + KS-CLF; lane 6, holo-HedE ACP + MAT + KS-CLF. For quantitative estimates of radiolabeling intensities of individual bands (errors 2%–3%), see text.

R1128 initiation module with the *hed* minimal PKS (*S. coelicolor* CH999/pAD281) led to the production of an isobutyryl primed product, AD281a (**19**). The structure of AD281a was confirmed by ^{1}H and ^{13}C NMR spectroscopic analysis (Table 3) as well as HSQC, COSY and HMBC NMR experiments (Supplemental Data). HR-ESI-MS confirmed that AD281a has the molecular formula $C_{24}H_{24}O_8$ (observed m/z 463.1369 [M + Na]$^+$, calcd. m/z 463.1371 for $C_{24}H_{24}O_8Na$). AD281a is an analog of SEK15 (Fu et al., 1994) and verifies two predictable features of the *hed* minimal PKS: (i) that it exercises chain length control by monitoring the length of the carbon chain backbone; and (ii) that it can be primed by the R1128 ACP to synthesize a C-24 backbone. Interestingly, AD281a shows C7-C12 cyclization pattern, which may be due to the aromatase activity of HedE.
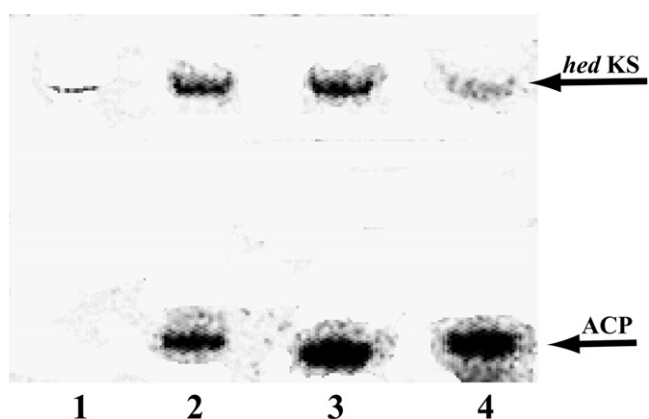

Figure 5. Autoradiograph of Gel Showing the Labeling of KS-CLF in the Presence of Alternative Hexanoyl Donors
Lane 1, [^{14}C]-hexanoyl-CoA; lane 2, [^{14}C]-hexanoyl-HedU; lane 3, [^{14}C]-hexanoyl-ZhuG; lane 4: [^{14}C]-hexanoyl-FrenJ.
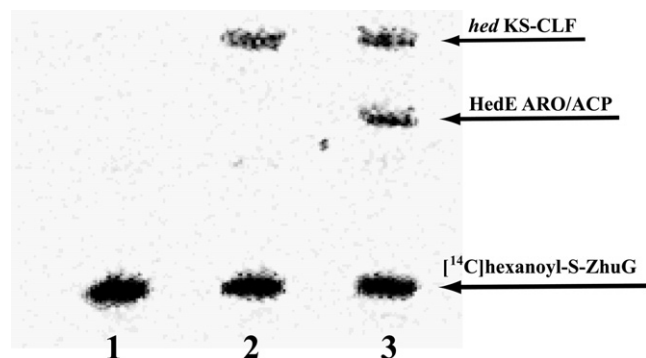
Figure 6. Autoradiograph of Gel Showing KS-CLF Catalyzed Labeling of Elongation Holo-ACP

Lane 1, [^{14}C]-hexanoyl-ZhuG and HedE ARO/ACP; lane 2, [^{14}C]-hexanoyl-ZhuG and *hed* KS-CLF; lane 3, [^{14}C]-hexanoyl-ZhuG, HedE ARO/ACP and *hed* KS-CLF.

DISCUSSION

The hedamycin PKS gene cluster reported by Thorson and coworkers illustrated a fundamentally new and unusual principle for priming a polyketide derived from a type II PKS (Bililign et al., 2004). Specifically, it was proposed that a type I PKS from the hedamycin gene cluster is involved in synthesizing the unsaturated primer unit. Our results have verified that the *hed* minimal PKS is indeed a dodecaketide synthase, although it can also synthesize undecaketides. Minimal PKS activity is not obligately dependent upon a functional initiation module. Unlike the R1128 PKS, the KSIII and AATE homologs of the hedamycin gene cluster do not appear to be involved in chain initiation; instead, hedamycin biosynthesis is primed by HedU, possibly in collaboration with HedT. Our data also shows that HedE is a bifunctional protein with ACP and ARO activity, and that the *hed* KR is similar to *act* KR. Both the *hed* ARO and the *hed* KR are able to modulate the chain length specificity of the KS-CLF, as illustrated by variable ratios of undecaketide and dodecaketide products in the presence or absence of these auxiliary PKS subunits. The *hed* KS-CLF heterodimer has specificity for its ACP partners (i.e., the HedU ACP domain during chain transfer and the HedE ACP domain during chain elongation), and shows a greater ability to discriminate against heterologous ACPs than most homologous enzymes investigated thus far. Coexpression of R1128 initiation module and the *hed* minimal PKS leads to the synthesis of isobutyryl primed polyketide product. Together, our results demonstrate that the hedamycin polyketide synthase is an interesting model system that warrants further genetic, biochemical, and structural investigations.

SIGNIFICANCE

Mechanistic analysis of unusual PKSs is essential to understand the molecular basis for polyketide natural product diversity. It has also enabled the engineered biosynthesis of "unnatural" natural products. The results of our in vitro and in vivo studies on the hedamycin PKS highlight its typical and atypical characteristics, and emphasize its rele-

vance to fundamental and applied studies involving type I and type II PKSs. In particular, our findings reported here have at least two implications worthy of further pursuit. First, structural and mechanistic studies are warranted to understand how the *hed* KS-CLF accommodates two ACP domains from HedU and HedE, while at the same time discriminating against an unusually wide range of heterologous ACP subunits. Second, now that the biosynthetic relevance of the type I-type II PKS interface in the hedamycin pathway has been firmly established, more detailed investigations into this unusual recognition interface promise to reveal fundamentally new opportunities for harnessing the catalytic potential of both major subclasses of PKSs to synthesize novel polyketides.

EXPERIMENTAL PROCEDURES

Materials

Radio-labeled coenzyme A (CoA) substrates were purchased from American Radiolabeled Chemicals (St. Louis, MO). FLAG peptide was purchased from Genscript (Piscataway, NJ). Corp. Anti-FLAG M2 monoclonal antibody, anti-FLAG M1 agarose affinity gel, and all other biochemicals, including unlabeled CoA substrates, were from Sigma-Aldrich, Inc. (St. Louis, MO). Hi-Trap Q anion-exchange column was from Amersham Biosciences (now GE Healthcare, Piscataway, NJ). Precast SDS-PAGE gels were from Invitrogen (Carlsbad, CA). PCR was performed using *PfuTurbo Hotstart* polymerase (Stratagene, La Jolla, CA), and amplimers were sequence verified prior to further use. All cloning steps were performed in *E. coli* XL1-Blue and *E. coli* TOP10. Unmethylated DNA was obtained by passaging plasmids through the methylase-deficient strain *E. coli* ET12567. Genomic DNA from *Streptomyces griseoruber* was prepared using QIAGEN Genomic DNA Purification Kit (Qiagen Corp., Valencia, CA). Protoplast preparation and PEG-assisted transformation were performed as described Kieser et al. (2000). *S. coelicolor* CH999 and *E. coli* strains BL21(DE3) or BAP1 were used for protein expression. Plasmids used for cloning included pCR-BLUNT (Invitrogen), pUC18 (New England Biolabs, Ipswich, MA) and pET28b (Novagen, Gibbstown, NJ). The deuterated solvents for NMR experiments were purchased from Cambridge Isotope Laboratories, Inc (Andover, MA) and all other solvents were purchased from Fisher Scientific (Bridgewater, NJ) at highest available grade.

Cloning of Hedamycin Genes

The *hed* PKS genes, *hedC*, *hedD*, *hedE* and *hedA*, were amplified by PCR using *S. griseoruber* genomic DNA as the template (Bililign, et al., 2004). The primers used were hedC_FP: GG*TCTAGAattaattaa***GGAGG**ACC*CATATGG*TCC GAGACACGCCCGCAGG and hedC_RP: GG*ACTAGT*TCATGCGGGCCGTC CCGGAGCCGT; hedD_FP: GG*TCTAGA***GGAGG**ACC*CAT*ATGGACTACAAG GACGACGACGACAAGATGAACGGCGACGCGCTGATCACGGGC and hedD_RP: GG*ACTAGT*TCAACCGCCAGGTCGCCGAGGCCG; hedE_FP: GG*TCTAGA*G **GAGG**ACC*CATATG*GGGCGGCTCCTGGACCTTGGAG and hedE_RP: GG*ACT AGT*TCAGGCGGCCTCGGCGACCTGGCGGTT; hedA_FP: GG*TCTAGA***GGA GG**ACC*CATATG*GTCCCGTCCCCAGACCGCCTTCGT and hedA_RP: GGAA TT*ACTAGT*TCAGTAGTTGCCCAGGCCGCCGCAGAC. Restriction sites are indicated in italics or in lowercase, ribosomal binding sites are in bold, and the underlined sequence corresponds to the FLAG tag. PCR amplimers were cloned into the pCR-BLUNT vector, and their DNA sequences were verified. The genes were then assembled as synthetic operons in pUC18 prior to transfer into a pRM5-derived shuttle vector. The first gene in each operon was inserted between the *XbaI* and EcoRI sites in pUC18; subsequent genes were inserted downstream following *SpeI/EcoRI* digestion of the vector and *XbaI/ EcoRI* digestion of the insert. To do so, we took advantage of the naturally occurring EcoRI site in the pCR-BLUNT vector. The complete operon was isolated by *PacI/EcoRI* digestion, and inserted between the *PacI/EcoRI* sites of the *S. coelicolor/E. coli* shuttle vectors pRM5 (which carries the *act* ketoreductase gene) or pSEK4 (which lacks this KR gene) (McDaniel et al., 1993a), displacing the *act* PKS genes (Table 2). For construction of plasmids that harbor
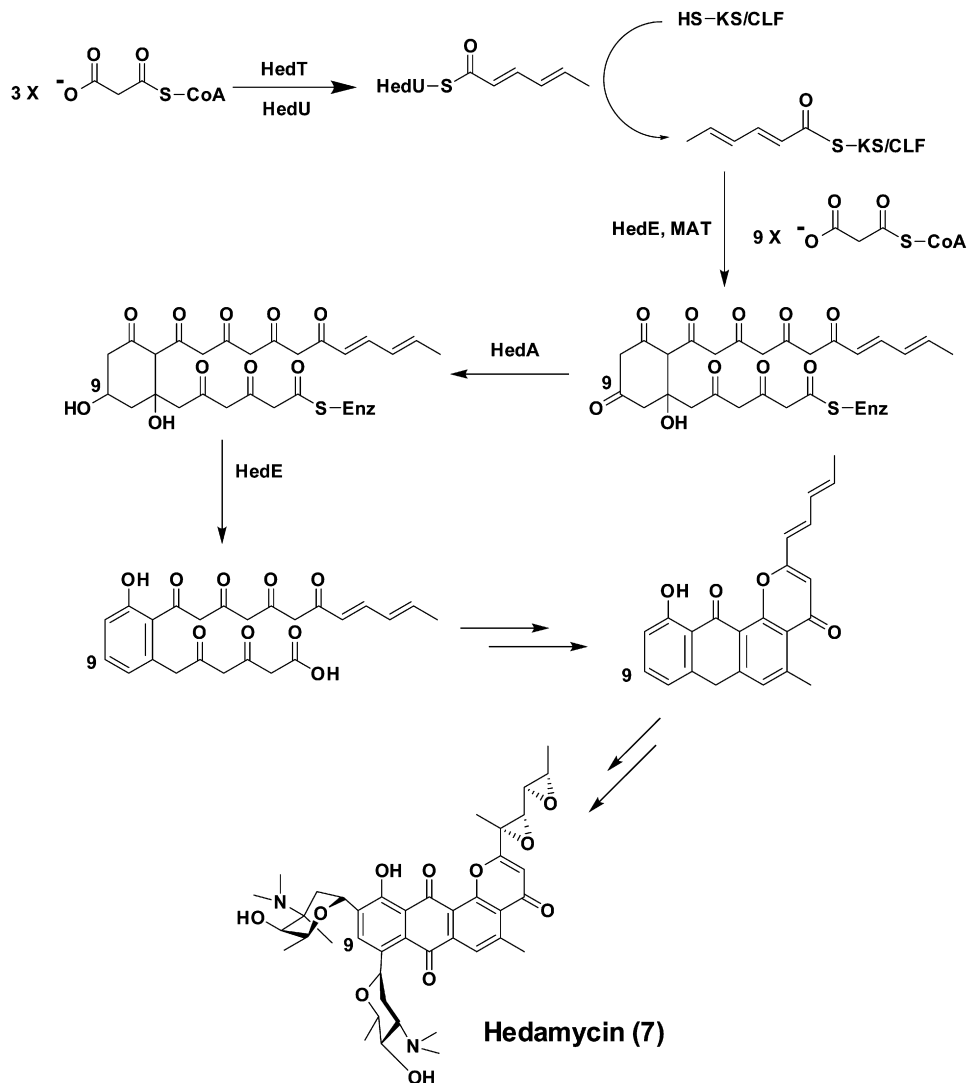
Figure 7. Proposed Pathway for Hedamycin Biosynthesis

R1128 PKS genes, pYT45 and pYT81 were used as cloning vectors (Tang et al., 2004b; 2004c). The resulting plasmids were introduced via protoplast transformation into *Streptomyces coelicolor* CH999 (McDaniel et al., 1993a) containing the plasmid pBOOST* (Hu et al., 2003).

Culture Conditions and Small-Scale Fermentation Analysis

The strains were grown on R5 agar plates (Kieser et al., 2000) containing 50 mg/l thiostrepton and 100 mg/l of apramycin at 30°C for 7 days. For HPLC analysis, the pigmented agar medium from 10-12 plates was mashed and extracted first with methanol/ethyl acetate (10:90) and then with acetic acid/ethyl acetate (1:99). The solvent was evaporated in vacuo and the residue was dissolved in methanol. The mixture was analyzed by reverse-phase HPLC outfitted with a UV-Vis detector at 280 and 410 nm, using an Apollo C_{18} column (250 mm × 4.6 mm). A linear gradient from 30% acetonitrile (MeCN) in water (0.1% TFA) to 55% MeCN in water (0.1% TFA) was used over 40 min with a flow rate of 1 mL/min. LC/MS of the same crude extract was performed by electrospray ionization (both positive and negative ionization). For precursor directed biosynthesis experiments, acyl-CoA precursors such as sodium propionate, DL-aminobutyric acid and norvaline were added while preparing the media at a concentration of 1-4 g/l when needed (Tang et al., 2004c).

Large-Scale Production, Isolation, and Characterization of Polyketide Products

For preparative scale studies, the above fermentation and crude extract preparation procedures were scaled up to 3-4 L of R5 agar medium. The extract was dried with toluene, and the solvent was removed in vacuo. The crude extract was flashed through a silica gel column using 1% acetic acid in ethyl acetate. The eluate was dried, redissolved in 3-4 ml methanol, and injected onto the preparative reverse-phase HPLC column (250 mm × 22 mm, C_{18} column, Vydac). Gradients used included 10%–40% MeCN in water (0.1% TFA) over 80 min or 20%–60% MeCN in water (0.1% TFA) over 100 min, with a flow rate of 5 ml/min. Purified fractions were dried in vacuo or by lyophilization. In cases where further purification was warranted, the relevant HPLC fraction was redissolved in 0.5-1 ml acetone and applied to a preparative TLC plate, which was developed in a suitable solvent system consisting of ethyl acetate and hexane with or without 1% acetic acid. ^{1}H and ^{13}C NMR spectra of purified polyketide products were recorded on Varian Inova 500. The ^{1}H NMR spectra were referenced at 2.49 ppm in DMSO-d_6. The ^{13}C NMR spectra were referenced at 39.5 ppm in DMSO-d_6. Single-bond [1]H-^{13}C, multiple-bond [1]H-^{1}H and [1]H-^{13}C connectivity was determined by HSQC, COSY and HMBC respectively on Varian Inova-600 NMR instruments. The ^{1}H and ^{13}C NMR assignments are summarized in Table 3 and Supplemental

Expression and Purification of the Hedamycin KS-CLF

S. coelicolor CH999/pAD211 was used to express and purify *hed* KS-CLF. The plasmid pAD211 encoded the KS, the CLF, the bifunctional HedE ACP/aromatase and the *act* KR. The CLF protein harbored a FLAG-tag at its N terminus to facilitate purification of the tightly associated KS-CLF heterodimer. Spores were inoculated into 50 ml R5 liquid medium, and grown for 2 days at 30°C. The seed culture (25 ml) was transferred to 0.5 l SMM medium containing 50 mg/l thiostrepton and 100 mg/l apramycin, and grown further with vigorous shaking for 48 hr. Mycelia were collected by centrifugation, resuspended in 25 ml disruption buffer (200 mM Na_2HPO_4, 200 mM NaCl, 2.5 mM DTT, 1.5 mM benzamidine, 2.5 mM EDTA, 2 mg/L pepstatin, 2 mg/L leupeptin, 30% v/v glycerol, pH 7.0), and lysed by sonication. The insoluble cell debris was removed by centrifugation. Subsequently, DNA was also removed by adding 0.2% polyethyleneimine (PEI) and centrifuging. The proteins in the resulting supernatant were precipitated by adding 30% $(NH_4)_2SO_4$ followed by 50% $(NH_4)_2SO_4$. The protein mixture in the 30%–50% $(NH_4)_2SO_4$ cut was redissolved in 30ml TBS buffer [50 mM Tris (pH 7.4), 0.15 mM NaCl and 10 mM $CaCl_2$], filtered through 0.45 μm filter, and loaded onto a column packed with anti-FLAG M1 agarose affinity gel (1 ml). The column was washed with 10 ml of TBS, and KS-CLF was eluted with 5 × 1 ml of TBS containing 100 μg/mL 3 × FLAG peptide. The eluent was concentrated and buffer exchanged into 100 mM NaH_2PO_4, 2 mM DTT, 2 mM EDTA, 20% Glycerol (pH 7.4), aliquoted, flash-frozen with liquid nitrogen, and stored at −80°C. The protein concentration was determined by the Bradford method with the Bio-Rad protein kit. The yield of *hed* KS-CLF was 1-2 mg/l culture volume.

Construction, Expression, and Purification of HedE and Its Derivatives

HedE is a unique PKS subunit that consists of a partial aromatase (ARO) domain at the N terminus, followed by an intact ARO domain and an ACP domain. Attempts to express the intact HedE protein in *E. coli* were unsuccessful. We therefore constructed two additional expression plasmids containing the ACP domain alone (identified by sequence analysis with other type II ACPs) and a bifunctional protein consisting of the intact ARO and ACP domains (ARO/ACP). The two genes were amplified using the primers hedE_ACP_FP: GGAATT*CATATG*ATGGCCGAACTGACCCTCGACGAGCTCAAG, hedE_ACP_RP: GGAATT*ACTAGT*TCAGGCGGCCTCGGCGACCTGGCGGTTGAC and hedE_ARO/ACP_FP: GGGG*CATATG*GCCGACACCGAGACCGTCTCCGGC, hedE_ARO/ACP_RP: GG*ACTAGT*GCGGGCGGCGTGCGGCGAGGCCCA. These PCR products were cloned into pET28b to yield pAD58 and pAD227, respectively. The genes were expressed in an engineered *E. coli* strain BAP1 (Pfeifer et al., 2001) to obtain the pantetheinylated (holo) forms of the corresponding proteins. Protein expression was induced at an O.D.$_{600nm}$ of 0.6 with 200 μM IPTG and allowed to proceed at 18°C for 18 hr. Cells were harvested and lysed by sonication. The proteins were initially purified on Ni-NTA resin (QIAGEN). After loading the protein, the Ni-NTA resin was washed with buffer containing 20 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4 (pH 8.0). The protein was then eluted with buffer containing 200 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4 (pH 8.0). This was followed by anion-exchange chromatography on a Hi-Trap Q column (from 0 to 100% of 1M NaCl in 60 min, proteins elute between 200 and 300 mM NaCl). Purified proteins were buffer exchanged into 100 mM sodium phosphate, 20% glycerol (pH 7.2) and stored at −80°C. The relative ratio of the holo and apo forms of ACP was quantified by LC-MS; in each case, the ACP was found to be nearly 100% in the holo form. Protein concentrations were determined by the Bradford method with the Bio-Rad protein kit. No expression of HedE was observed however from *E. coli* BAP1 or *E. coli* BL21(DE3) strains. Typical yield of each protein was between 10-12 mg/l.

Expression and Purification of Other Acyl Carrier Proteins

A variety of other ACPs from initiation and elongation modules of type II PKSs were used in this study. All of the elongation ACPs were expressed and purified from *E. coli* BAP1 to obtain exclusively the proteins in holo form. The ACPs were then purified by Ni-NTA affinity column. Expression strains *E. coli* BAP1/pGS1, BAP1/pESM11 (Meadows and Khosla, 2001), and BAP1/pAD191 were used to obtain FrenN (the ACP from the elongation module of the frenolicin PKS), ZhuN (ACP from the elongation module of the R1128 PKS) and FdmH (ACP from the fredericamycin PKS), respectively. The yield of each protein was in the range of 15-20 mg/l.

To prepare acyl-ACP forms of FrenJ (from the initiation module of the frenolicin PKS) and ZhuG (from the initiation module of the R1128 PKS), the proteins were first expressed and purified from *E. coli* BL21(DE3) cell line to obtain apo-ACPs exclusively. ZhuG was from BL21(DE3)/pESM10 *E. coli* expression strain (Meadows and Khosla, 2001), whereas FrenJ was from BL21(DE3)/pYT21(Tang et al., 2003a).

To investigate the role of HedU in hedamycin biosynthesis, the terminal ACP domain of this type I PKS protein was amplified. The boundary of this ACP domain was identified by aligning the sequence of HedU with module 1-6 of DEBS PKS (Chen et al., 2007). The ACP domain was amplified by PCR using the primers hedUACP_FP: GGAATT*CATATG*CGGCTCGCCGGCCGGTCCGAGGCGGAG and hedUACP_RP: GGAATT*ACTAGT* TCATGCGGGGTGCAGCAGTTCCGCGAGGTG. The PCR product was digested with NdeI/SpeI and ligated into a pET28b derivative to yield pAD64. The His$_6$-tagged protein was expressed in *E. coli* strain BL21(DE3) to obtain apo-form of the protein. Protein expression was induced at an O.D.$_{600nm}$ of 0.6 with 200 μM IPTG and allowed to proceed at 20°C for 20 hr. The purification method was similar to that for the HedE ACP. The protein yield was 12 mg/l.

Expression and Purification of MAT and Sfp Proteins

S. coelicolor MAT was expressed from *E.coli* BL21(DE3)/pGFL16 and purified as described previously (Carreras and Khosla, 1998). To prepare acyl-ACP proteins, the broad specificity phosphopantetheinyl transferase Sfp (Lambalot et al., 1996; Quadri et al., 1998) was purified and used as described earlier (Dreier and Khosla, 2000). For example, conversion of an apo-ACP to [^{14}C]-hexanoyl-S-ACP was carried out in buffer containing sodium phosphate, pH 7.0 and 10 mM $MgCl_2$ (Tang et al., 2004a). Apo-ACP, Sfp and [^{14}C]-hexanoyl CoA were added to the final concentrations of 120, 10, and 300 μM, respectively, in a reaction volume of 200 μl. After overnight incubation at room temperature, the mixture was diluted to 2.5 ml with the reaction buffer and loaded onto a PD-10 desalting column (Amersham, now GE Healthcare) that had been pre-equilibrated with the reaction buffer. Acyl-ACPs were eluted within the first 3.5 mL, and concentrated with 3000 Da MWCO spin column (Amicon Ultra, Millipore) to 20 μl. Then 1 μl of the final concentrate was applied to a SDS-PAGE gel, and the amount of radioactivity was quantified by autoradiography to calculate the effective concentration of the hexanoyl-ACP. Labeled acyl-ACP proteins were stored at −20°C.

Detection of Covalently Bound Protein Intermediates in Polyketide Biosynthesis

To investigate chain initiation and elongation in vitro, selected protein combinations were incubated with radio-labeled substrates and analyzed via radio-SDS-PAGE. For example, a PKS reaction mixture (12 μl) contained 200 μM [^{14}C]malonyl CoA (55Ci/mol), 10 μM the *hed* KS-CLF, 40-50 μM holo-ACP, and 1-5 μM MAT in a buffer containing 100 mM sodium phosphate, pH 7.3, 2 mM EDTA, 2 mM DTT. Following incubation at room temperature for 8-14 min, the reaction was quenched with 6 μl of non-reducing sample buffer (0.1% bromophenol blue, 1% SDS, 40% glycerol), and loaded directly onto a 4%–12% SDS-PAGE. Following staining, dried gels were subjected to autoradiography.

To assay the ability of *hed* KS-CLF to accept nonacetyl primer units, the purified heterodimer was incubated with alternative [^{14}C]-hexanoyl-S-ACPs or [^{14}C]-hexanoyl CoA. 10 μM KS-CLF was incubated with 2 μM [^{14}C]-hexanoyl-S-ACP (27 Ci/mol) or 100 μM [^{14}C]-hexanoyl CoA (27Ci/mol) in a reaction buffer (12 μl) containing 100 mM sodium phosphate, pH 7.3, 2 mM EDTA, 2 mM DTT for 30 min at RT. The reaction was quenched by adding 6 μl of non-reducing sample buffer, and loaded directly onto 4%–12% SDS-PAGE. Following staining, dried gels were subjected to autoradiography.

To investigate whether the *hed* KS-CLF can catalyze transfer of a nonacetyl primer from an ACP of an initiation module to the HedE (elongation module) ACP, 10 μM KS-CLF was incubated with 2 μM of [^{14}C]hexanoyl-S-ZhuG (27 Ci/mol) in reaction buffer (12 μl) containing 100 mM sodium phosphate, pH 7.3, 2 mM EDTA, 2 mM DTT for 30 min at room temperature. Thereafter,

50 μM HedE ACP/aromatase protein was added to the mixture and incubated for another 15 min. As a control, 2 μM of [^{14}C]-hexanoyl-S-ZhuG (27 Ci/mol) was incubated separately with the *hed* KS-CLF or HedE for the same amount of time.

SUPPLEMENTAL DATA

Supplemental Data include five tables and two figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00369-X.

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