CHEMBIOCHEM

DOI: 10.1002/cbic.200800332

A Polylinker Approach to Reductive Loop Swaps in Modular Polyketide Synthases

Laurenz Kellenberger,[a] Ian S. Galloway,[a] Guido Sauter,[a] Günter Böhm,[b] Ulf Hanefeld,[b] Jesús Cortés,[a] James Staunton,[b] and Peter F. Leadlay*[a]

Multiple versions of the DEBS 1-TE gene, which encodes a truncated bimodular polyketide synthase (PKS) derived from the erythromycin-producing PKS, were created by replacing the DNA encoding the ketoreductase (KR) domain in the second extension module by either of two synthetic oligonucleotide linkers. This made available a total of nine unique restriction sites for engineering. The DNA for donor "reductive loops," which are sets of contiguous domains comprising either KR or KR and dehydratase (DH), or KR, DH and enoylreductase (ER) domains, was cloned from selected modules of five natural PKS multienzymes and spliced into module 2 of DEBS 1-TE using alternative polylinker sites. The resulting hybrid PKSs were tested for triketide production in vivo. Most of the hybrid multienzymes were active, vindicating the treatment of the reductive loop as a single structural unit, but yields were dependent on the restriction sites used. Further, different donor reductive loops worked optimally with different splice sites. For those reductive loops comprising DH, ER and KR domains, premature TE-catalysed release of partially reduced intermediates was sometimes seen, which provided further insight into the overall stereochemistry of reduction in those modules. Analysis of loops containing KR only, which should generate stereocentres at both C-2 and C-3, revealed that the 3-hydroxy configuration (but not the 2-methyl configuration) could be altered by appropriate choice of a donor loop. The successful swapping of reductive loops provides an interesting parallel to a recently suggested pathway for the natural evolution of modular PKSs by recombination.

Introduction

Modular type I polyketide synthases (PKSs), for example the 6-deoxyerythronolide B synthase (DEBS) responsible for synthesis of the aglycone core of the macrolide antibiotic erythromycin A, are giant, multifunctional enzymes that catalyse the biosynthesis of structurally complex and clinically important polyketide products.[1–3] Polyketide chain formation closely resembles fatty acid biosynthesis, in which a starter unit and successive extender units derived from the CoA esters of simple carboxylic acids are condensed together to create a chain. As in fatty acid chain formation, the intermediates are not released into solution. In contrast to fatty acid synthases (FASs),[4] modular PKSs utilise a wider variety of starter and extender units, and the β-keto functionality created after each condensation step is not necessarily fully reduced before the next cycle of chain extension, leading to a far greater chemical and stereochemical diversity in the products.

The sequencing of the genes for DEBS (and for many other such PKSs) has revealed that each cycle of chain extension is catalyzed by a different set or "module" of active sites. A typical module contains a ketosynthase (KS) domain that catalyses the formation of a carbon-carbon bond. The acyltransferase (AT) domain recruits the chain extension unit, normally from either malonyl-CoA or methylmalonyl-CoA. The acyl carrier protein (ACP) cooperates in the carbon-carbon bond formation to form a β-ketoester. Depending on the module, there may be additional activities present: a β-ketoacyl reductase (KR) domain catalyses reduction of the initially formed β-ketoester to a β-hydroxyester; a dehydratase (DH) domain dehydrates the β-hydroxyester; and an enoyl reductase (ER) domain reduces the double bond. This set of contiguous domains, which modifies the oxidation state of a freshly introduced extension unit, is referred to here as a "reductive loop", and defined as the part of each module that lies between the C terminus of the AT domain and the N terminus of the ACP domain (Figure 1). Initially identified by limited proteolysis experiments on purified DEBS proteins,[5,6] recent studies[7–9] have confirmed that this entire region adopts a specific tertiary and quaternary structure. Intriguingly, recent analysis of recombinational events implicated in the natural evolution of modular PKSs has highlighted the termini of reductive loops as hotspots for such recombination.[10]

Success in engineering changes in the oxidation level of a polyketide product initially came through deletion of portions of the reductive loops of DEBS.[11–12] Similarly, the replacement of the DEBS KR2 domain by the inactive KR3 domain led suc-

[a] *Dr. L. Kellenberger, Dr. I. S. Galloway, Dr. G. Sauter, J. Cortés, Prof. P. F. Leadlay*
Department of Biochemistry, University of Cambridge
80 Tennis Court Road, Cambridge CB2 1GA (United Kingdom)
Fax: (+44) 1223-766002
E-mail: pfl10@mole.bio.cam.ac.uk

[b] *G. Böhm, Dr. U. Hanefeld, Prof. J. Staunton*
University Chemical Laboratory, University of Cambridge
Lensfield Road, Cambridge CB2 1EW (United Kingdom)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

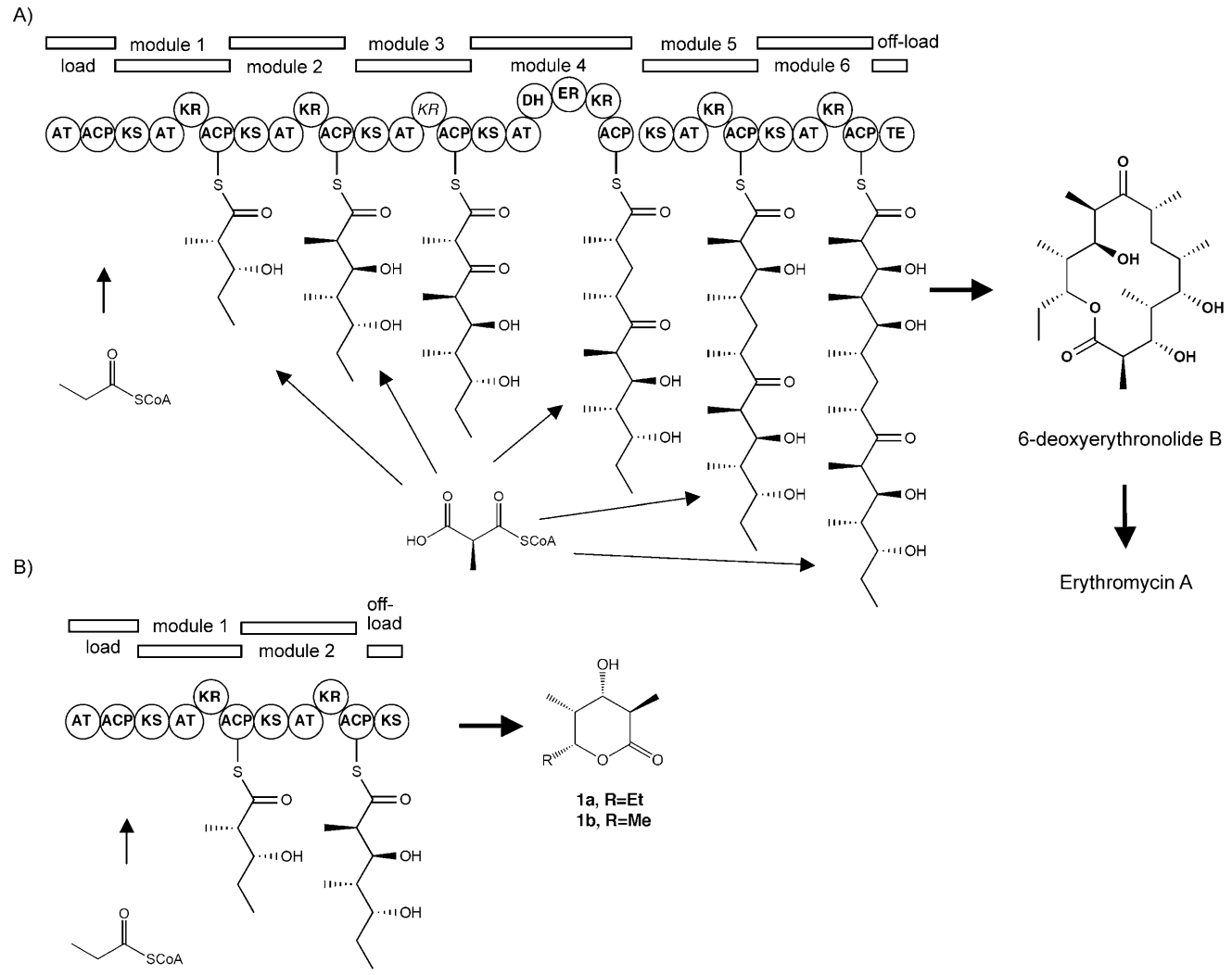


Figure 1. Domain and module organisation of A) the erythromycin PKS multienzyme and of B) the derived triketide synthase DEBS 1-TE. Within each of the three subunits (DEBS 1, DEBS 2 and DEBS 3) and in DEBS 1-TE, different enzymatic domains are denoted by circles. KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; ACP, acyl carrier protein; TE, thioesterase/cyclase. The italicised KR domain in DEBS module 3 is inactive.

cessfully to a keto-product.[13] The introduction of domain swapping as a method to create productive hybrid polyketide synthases between different natural PKSs[14,15] has prompted many subsequent attempts to swap reductive enzymes, with encouraging but mixed results; in some cases the predicted change in oxidation level was seen,[16–19] but in other cases one or more of the heterologous enzymes failed to function, leading either to a product with higher oxidation level than predicted, or no product at all.[17–20] Many of these swaps used the same points in the sequence at which to splice the incoming domains. Attempts to generate comprehensive libraries of polyketides through loop swapping at a single set of splice points have also fallen short, with some products obtained in only trace amounts.[21] Improvements in reductive loop swapping would therefore be of considerable practical significance for engineered biosynthesis of high-value, complex polyketides.

Another central issue in the mechanism of polyketide chain growth on the PKS is that the methyl centres at C-2 and the hydroxy centres at C-3 generated in many newly added chain extension units can both have either a *R* or *S* configuration. As early as 1965, position-specific configurational homologies were noted among natural macrolide polyketides and codified as the empirical Celmer's rules.[22] Understanding how this stereochemical outcome is controlled is important for attempts to manipulate PKS catalysis to create novel polyketide products. In vitro studies with an engineered and purified bimodular PKS, DEBS 1-TE, have already revealed the crucial elements of the molecular basis of this control, and hence of Celmer's rules. DEBS 1-TE (Figure 1) was created by relocating the chain-terminating thioesterase from DEBS 3 to the C terminus of bimodular DEBS 1 to promote chain release at the triketide stage giving a δ-lactone.[23]

Although the two methyl-bearing centres in this product have opposite configurations, work with DEBS 1-TE has shown that the acyltransferase (AT) domain does not determine the stereochemical outcome of chain extension. The AT domains of DEBS bind only (2*S*)-methylmalonyl-CoA in vitro and not the

2*R*-isomer.[24] Only the (2*S*) isomer is used as a substrate by DEBS 1-TE.[25] There is an additional obligatory epimerisation step in module 1, after condensation, to produce the substrate for ketoreduction.[26–29] The stereochemical outcome therefore essentially depends on the interplay between the properties of individual KS and KR domains. In any chimaeric extension module that generates a methyl centre at C-2 and a hydroxy centre at C-3, if the product of condensation is exactly that stereoisomer of the 2-methyl-3-ketoacyl thioester intermediate which matches the normal preference of the incoming KR, then the heterologous KR often imposes the stereochemical course of its normal reaction on the new substrate.[18] However, other evidence suggests that the competition between alternative pathways for ketoreduction is finely balanced and can be perturbed. For example, this can occur if a KR within a chimaeric extension module is presented only with a single stereoisomer of the 2-methyl-3-ketoacyl thioester intermediate against which it is inactive.[30] Conversely the KR in its new context may be given access to both 2-methyl stereoisomers, which allows the intrinsic KR selectivity to dictate the outcome at both C-2 and C-3 stereocentres.[30]

We present here the results of introducing synthetic oligonucleotide polylinkers into the DNA encoding DEBS 1-TE in place of the KR_2 domain, and their use in splicing heterologous "reductive loops" to obtain hybrid PKSs that synthesise triketide lactones differing in oxidation level or (where the C-3 hydroxyl is retained) in C-3 alcohol and C-2 methyl stereochemistry. Our findings shed further light on the substrate specificity and stereochemical course of catalysis in module 2 of DEBS and in the other extension modules whose reductive loops have been assayed. More generally, the results show that worthwhile optimisation of hybrid modular PKSs can be achieved by trying alternative donor "reductive loops," as well as alternative splice sites within such polylinkers.

Results and Discussion

Replacement of the reductive loop in module 2 of DEBS1-TE by a short polypeptide linker

The reductive loop[6] of module 2 of DEBS1-TE comprises a functional KR domain and a large domain proposed to act either as a structural subdomain of the KR[7] or to contribute to stabilisation of the PKS homodimer[8] (Figure 2). The idea of treating reductive loops from PKS extension modules as integral units in constructing hybrid PKSs was suggested both by detailed comparisons among published modular PKS sequences and by the results of limited proteolysis of purified DEBS proteins,[5,6,31] showing that these pieces of the structure were often released early and intact. Subsequent work (see below) has amply vindicated this choice. In order to replace the DNA encoding this region by a polylinker, a version of the DEBS 1-TE gene was first engineered in which the reductive loop region was flanked by a unique 5′ AvrII restriction site and by a unique 3′ HpaI site, as described in the Experimental Section. Plasmid pJLK07, a pCJR24-derived[32] plasmid housing this engineered gene, was then cut with AvrII and HpaI, and its backbone was ligated to a 122 bp AvrII-HpaI dsDNA fragment, which was created by annealing together a pair of synthetic oligonucleotides. These were designed to conserve the amino

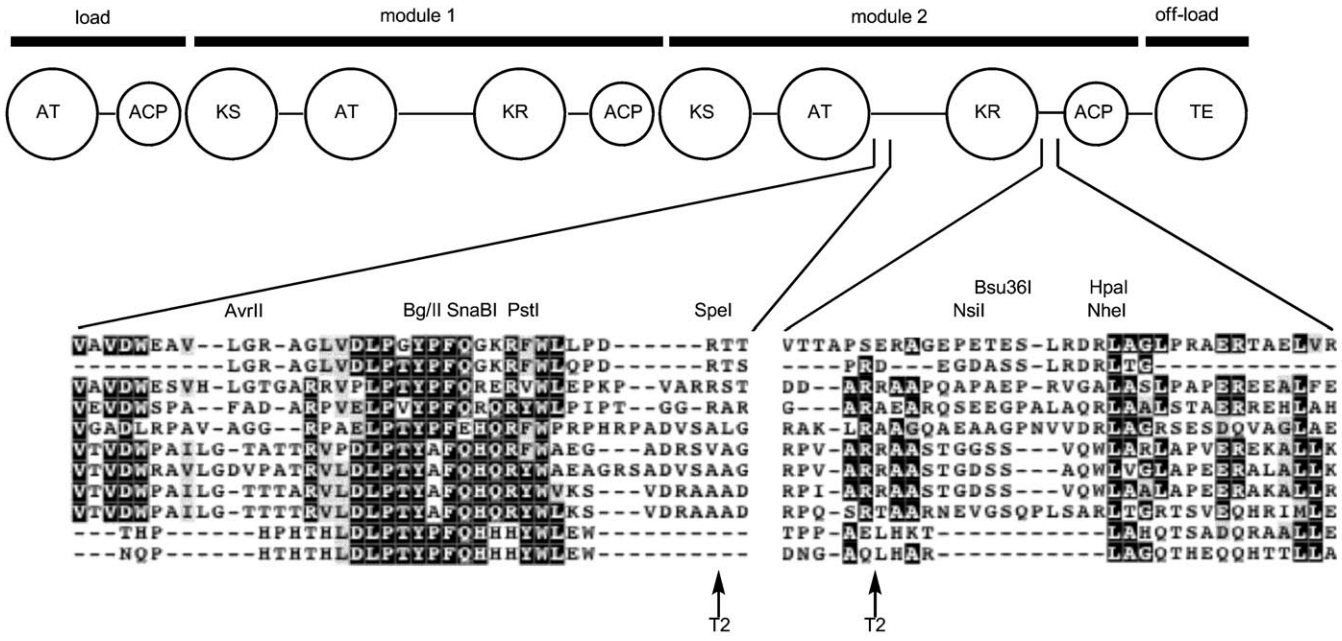


Figure 2. Design of a polylinker to replace the reductive loop (including the KR domain) of extension module 2 of DEBS1-TE. The alignment of modules from typical PKS extension modules shows the sequence flanking each reductive loop (C-terminal of the AT domain and N-terminal of the ACP domain). The oligonucleotide polylinker included the unique restriction sites AvrII, BglII, SnaBI, PstI, SpeI, NsiI, Bsu36I, NheI and HpaI, and the peptide linker sequence encoded by this polylinker is included in the alignment, which was created using ClustalW and Boxshade. Because the NheI and HpaI sites overlap, two versions of the polylinker were created, with either HpaI or NheI at this position. The arrows labeled T2 indicate the extent of the reductive loop in ery (DEBS) module 1, as shown by previous limited proteolysis experiments.[5]

acid sequences flanking the excised domains while introducing further unique restriction sites into the coding DNA (Figure 2). The resulting DEBS 1-TE gene, housed in plasmid pJLK114, contained a polylinker with unique sites for eight restriction enzymes. Some can be found in "linker" regions of low sequence similarity between different PKS modules, and others are in highly conserved regions within the AT domains of different PKS modules (Figure 2). A second version of the polylinker was obtained by replacing the unique Bsu36I-XbaI DNA fragment in pJLK114 with an identical fragment containing a unique NheI site in place of the HpaI site, which created plasmid pJLK117 (Experimental Section). By using a total of five restriction sites 5′ and four sites 3′ of any inserted "reductive loop" DNA, a wide choice of splice sites could be tried. The amino acid sequence of the peptide encoded by the polylinker is also given in Figure 2, showing that the incorporation of multiple restriction sites introduces only minimal changes to the amino acid sequence.

Production of triketide lactones of altered oxidation state by reductive loop insertion into the polylinker region of the engineered DEBS1-TE gene

Cloned DNA encoding the reductive loop regions of module 2 of DEBS (KR, control), module 10 of the rapamycin-producing PKS (RAPS, DH-KR) and module 13 of RAPS (DH-ER-KR) was spliced into the polylinker region of either pJLK114 or pJLK117, using different splice sites. The resulting plasmids were integrated into *Saccharopolyspora erythraea* JC2, a strain in which essentially all the erythromycin PKS genes have been deleted.[23] The resulting recombinants were grown on agar-based media as described in the Experimental Section. Their triketide products were extracted and analysed by GC-MS and electrospray mass spectrometry, and compared to authentic samples of each stereoisomer obtained by standard synthetic routes.[33–35] Figure 3 illustrates the GC separation of four such standard triketides. In all of these experiments, triketides were obtained in which either a propionate (**a** forms) or an acetate (**b** forms) had been used as starter unit. The molar ratio of the **a** to **b** forms in the products was between 2:1 and 4:1, which is in agreement with the results of previous studies.[23] The results of fermentation of the recombinant strains are summarised in Tables 1 and 2.

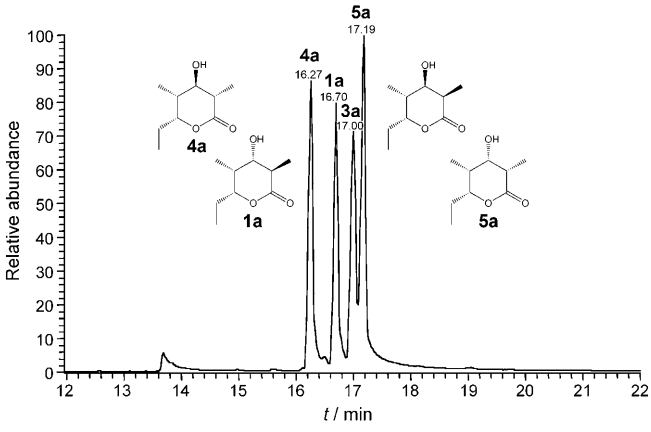
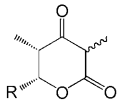
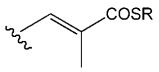
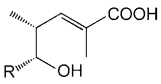
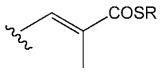
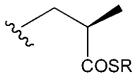
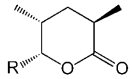
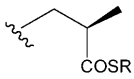
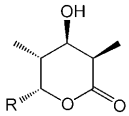
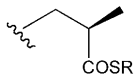
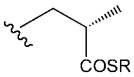
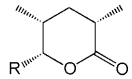


Figure 3. GC-MS separation and identification of chiral triketide lactones. An equimolar mixture of synthetic standard samples[33–35] of each of the four diastereoisomeric 3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lactones **1a**, **3a**, **4a** and **5a** was separated by GC and components were identified by mass spectrometry, as detailed in the Experimental Section and in the Supporting Information (Figure S1).

The DEBS 1-TE bearing the DEBS KR_2 domain on a SpeI-NsiI fragment (pJLK25) produced the expected triketide lactones **1a** and **1b** in unoptimised total yields (80–100 mg L^{-1}; Table 2) comparable to those (~100 mg L^{-1}) obtained from the parent DEBS 1-TE multienzyme under the same conditions; this shows that the four amino acid changes in the linker region flanking KR_2 (Figure 2) do not dramatically alter enzyme activity. The plasmids pJLK114 and pJLK117 containing no reductive loop were also used to transform *S. erythraea*, and the products of fermentation of the recombinant strains were in each case the 3-ketolactones **2a** and **2b** (Table 1), in significant though reduced yield (8–13 mg L^{-1}). These results confirm previous indications that substantial[11] or total[21,29] deletion of a KR domain within a modular PKS does not inactivate polyketide chain synthesis, and are consistent with the proposal that KR domains do not contribute to the dimeric core in PKS multienzymes.[5,6,8] Fermentation of recombinant *S. erythraea* (pJLK29), housing a DEBS 1-TE variant in which the reductive loop of module 10 of RAPS (KR and DH domains) had been inserted in the linker gave as predicted the open chain triketides **6a** and **6b** (analysed as their methyl esters). This is consistent with results reported previously,[21,36,37] in which the normal intermediate in PKS-catalysed reduction is the *trans*- (or *Z*-) isomer of the 2-enoyl thioester. In contrast, when a similar donor loop from RAPS module 4 (pJCR4; KR, DH and ER domains) was inserted between the AvrII and HpaI sites, only trace amounts of **6a** and **6b** were seen, accompanied by traces of ketolactones **2a** and **2b** (Table 1). Similarly, fermentation of *S. erythraea* (pJLK28), which contained a DEBS 1-TE incorporating the reductive loop of module 13 of RAPS (KR, DH and ER domains) as a BglII-NsiI fragment, produced only the predicted fully reduced lactones **7a** and **7b**, which is again consistent with results of previously published work.[16,17,21] In contrast, when the same donor reductive loop was introduced into DEBS1-TE as an AvrII-HpaI fragment in *S. erythraea* (pJLK27), the major products of a typical fermentation were not only **7a** and **7b** (40 mg $L)^{-1}$, but also the 3-ketolactones **2a** and **2b** (approximately 12 mg L^{-1}) and hydroxylactones **3a** and **3b** (about 3 mg L^{-1}). Introduction of RAPS module 1 reductive loop (KR, DH and ER domains) as a BglII-NheI fragment (pJCR1) gave similar levels of **7a** and **7b** and of **2a** and **2b**, but no hydroxylactones (Table 1). Introduction of the reductive loop from ery (DEBS) module 4 (KR, DH and ER domains) as a BglII-Bsu36I fragment gave the isomers **8a** and **8b** (Table 1) with C-2 configurations altered from those observed in **7a** and **7b**; this is expected based on their function in the native context. In the hybrid DEBS 1-TE constructs, reduction of ACP-bound triketide thioesters competes directly with TE-catalysed hydrolysis or cyclisation, and thus production of incompletely reduced triketides is a sensitive measure of less-efficient catalysis by the

Table 1. Changes in oxidation state as a consequence of reductive loop exchanges in module 2 of DEBS 1-TE.

Donor loop	Normal function	Major products	Minor products
Polylinker AvrII-HpaI (pJLK114)		 **2a**, **2b** (13 mg L^{-1})	
Polylinker AvrII-NheI (pJLK117)		**2a**, **2b** (8 mg L^{-1})	
RAPS mod10 BglII-NheI (pJLK29)		 **6a**, **6b** (72 mg L^{-1})	**2a**, **2b** (traces)
RAPS mod4 AvrII-HpaI (pJCR4)		**6a**, **6b** (traces)	**2a**, **2b** (traces)
RAPS mod1 BglII-NheI (pJCR1)		 **7a**, **7b** (40 mg L^{-1})	**2a**, **2b** (8 mg L^{-1})
RAPS mod13 AvrII-HpaI (pJLK27)		**7a**, **7b** (41 mg L^{-1})	 **3a**, **3b** (3 mg L^{-1}) + **2a**, **2b** (12 mg L^{-1})
RAPS mod13 BglII-NsiI (pJLK28)		**7a**, **7b** (> 30 mg L^{-1})	
DEBS mod4 BglII-Bsu36I (pJLK41)		 **8a**, **8b** (5 mg L^{-1})	**2a**, **2b** (traces) + **3a**, **3b** (traces)

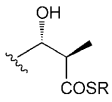
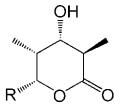
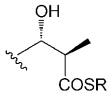
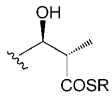
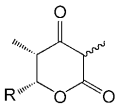
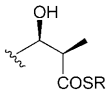
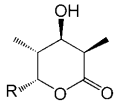
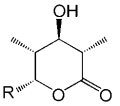
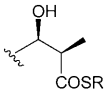
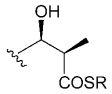
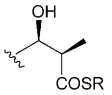
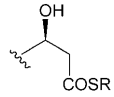
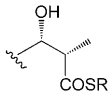
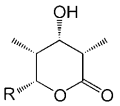
reducing enzymes. These results taken together serve to show that choice of splice sites, though usually dictated by convenience to avoid sites also present in the donor DNA, may alter protein-protein interactions in a way that reduces the effectiveness of reduction. It will always therefore be prudent to test two or more sets of splice sites.

The (2*R*,3*R*) configuration of the triketide lactones **3a** and **3b**, which are recovered as byproducts in some of these experiments, reveals the likely stereochemical course of ketoreduction by the KR in module 4 of DEBS and in module 13 of RAPS, both of which are normally obscured. The finding of the (2*R*,3*R*) isomer in each case is in full accordance with predictions for the direction of ketoreduction based on the presence of distinctive KR active site sequence motifs.[38,39] It also implies that the subsequent DH-catalysed dehydration proceeds as a *syn* elimination, exactly as for fatty acid synthase.[40] This agrees with and extends previous findings on the likely course of reduction in the comparable module 2 of the pikromycin-producing PKS.[41]

Production of triketide lactones of altered hydroxyl configuration at C-3 by reductive loop insertion into the polylinker region of the engineered DEBS1-TE gene

Reductive loops containing only an active KR domain were cloned into the polylinker site in the modified DEBS 1-TE gene. These donors were selected to include reductive loops from extension modules, which in their native PKS context provide various stereochemical outcomes at the 3-hydroxy- and (where this substituent was present) 2-methyl positions of the product acyl thioester (Table 2). For example, the reductive loop of DEBS module 5, which in its native context gives a (2*R*,3*S*) configuration in the alcohol product, was cloned into AvrII-HpaI sites (in *S. erythraea* JC2, pJCE5), and the fermentation of the resulting strain gave comparable yield and identical products to the control derived from DEBS module 2. This outcome makes a telling contrast to the results of previous in vivo work,[30] in which KS_5 was replaced by the DEBS loading module and KS_1 in DEBS 3. The products of that hybrid multienzyme included not only the normal products of DEBS3, but also an aberrant triketide lactone. Unambiguous determination of the stereochemistry at all chiral centres suggested that the

Table 2. Changes in configuration as a consequence of reductive loop exchanges in DEBS 1-TE.

Donor loop	Normal function	Major product	Minor products
DEBS mod2 SpeI-NsiI (pJLK25)		 **1a**, **1b** (100 mg L)$^{-1}$	
DEBS mod5 AvrII-HpaI (pJCE5)		**1a**, **1b** (100 mg L)$^{-1}$	
DEBS mod1 AvrII-HpaI (pJCE1)		none	 **2a**, **2b** (traces)
AVES mod1 BglII-NheI (pJLK30)		 **3a**, **3b** (25 mg L^{-1})	 **4a**, **4b** (traces)
AVES mod1 PstI-Bsu36I (pGMS2)		**3a**, **3b** (17 mg L^{-1})	**2a**, **2b** (5.5 mg L^{-1}) + **4a**, **4b** (traces)
TYLS mod1 BglII-NheI (pJLK35)		**3a**, **3b** (150 mg L^{-1})	**2a**, **2b** (12 mg L^{-1}) + **4a**, **4b** (traces)
RIFS mod8 SnaBI-NheI (pJCR8)		**3a**, **3b** (31 mg L^{-1})	**2a**, **2b** (19 mg L^{-1})
AVES mod2 BglII-NheI (pJLK31)		**3a**, **3b** (30 mg L^{-1})	**4a**, **4b** (traces)
RIFS mod7 SnaBI-NheI (pJCR7)		**2a**, **2b** (27 mg L^{-1})	 **5a**, **5b** (traces)

presence of KS_1 had induced epimerisation of the initially formed 2*R* isomer to the (2*S*) stereoisomer of the 2-methyl-3-oxopentanoyl-ACP intermediate, leading to "incorrect" processing not only in module 5 but also in module 6.[30] In the present case, bringing KR_5 into a chimaeric module with KS_2 did not lead to any loss of stereospecificity or stereoselectivity; this supports a model for catalysis[26,27,30] in which DEBS KS_2 differs from KS_1 in that it cannot catalyse the epimerisation of the (2*R*)-2-methyl-3-ketopentanoyl-ACP intermediate, or cannot do so fast enough to compete with ketoreduction. This experiment also shows that the ACP-tethered diketide is reduced with full fidelity by KR_5, even though previous in vitro studies have shown that recombinant purified KR_5 reduces the (untethered) racemic substrate (2*RS*)-2-methyl-3-oxopentanoic acid *N*-acetylcysteamine thioester to give three stereoisomeric products. Of these, only 25% is the expected (2*R*,3*S*) isomer. The remaining balance corresponds to 70% of the (2*S*,3*S*) isomer (altered at the methyl group) and 5% of the (2*R*,3*S*) isomer (altered at both centres).[42] The present data confirm the crucial importance of tethering to ACP for the stereocontrol of ketoreduction in modular PKS enzymes.[43,44]

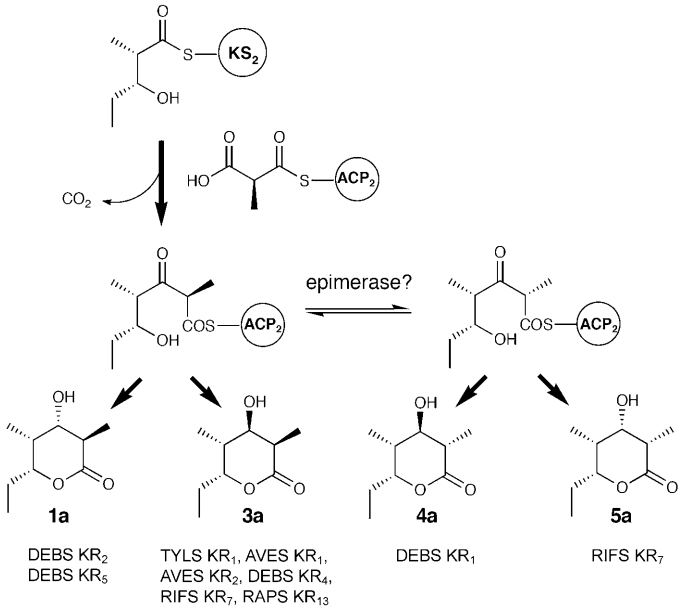
The *tyl* module 1 reductive loop from the PKS (TYLS) for the 16-membered macrolide tylosin of *Streptomyces fradiae*[42] governs, in its native context, the production of the (2*R*,3*R*) isomer of 2-methyl-3-oxopentanoic thioester (Table 2). This loop was introduced as a BglII-NheI fragment (Experimental Section). Fermentation of the corresponding recombinant strain *S. erythraea* (pJLK35) gave almost exclusively and in excellent yield the triketide lactones **3a** and **3b**, with (2*R*,3*R*) configuration as predicted (Table 2). The same compounds were produced from insertion of the reductive loop from module 1 of the PKS (AVES) for the anthelminthic avermectin.[46,47] Insertion of this

loop into the same BglII-NheI sites (pJLK30) also gave specific production of **3a** and **3b**, albeit at lower levels, while insertion of the AVES module 1 loop into PstI-Bsu36I (pGMS2) splice sites gave the same products accompanied by low yields of ketolactones **2a** and **2b** (Table 2). Introduction of the reductive loop from module 8 of the rifamycin-producing PKS (RIFS),[48] into SnaBI-NheI sites (pJCR8) gave comparable results. Interestingly, although AVES module 2 KR does not normally reduce a ketothioester bearing a 2-methyl substituent, insertion of the loop from this module as a BglII-NheI fragment (pJLK31) also gave reasonable yields of **3a** and **3b**. The only hybrid in the present study (not shown in Table 2) that gave no product at all was one in which the same AVES module 2 reductive loop was introduced as a SnaBI-Bsu36I fragment (pGMS4).

Several of the hybrid DEBS1-TE fermentations that produced the (2*R*,3*R*)-triketide lactones as major products gave traces of the diastereoisomeric (2*S*,3*R*) compounds **4a** and **4b** (Table 2). At face value, this means that a very small amount of the epimerised (2*S*)-2-methyl-3-oxopentanoic acid thioester intermediate is produced in these chimaeric modules. A possible explanation is that in these chimaeric modules chemical epimerisation occurs to a small extent because of imperfect sequestration of the intermediate. In sharp contrast, however, were the results from introduction of two other reductive loops from DEBS module 1 and from RIFS module 7.[48] Each of these contains a KR domain that is expected to be capable of efficient reduction of the epimerized (2*S*) isomer of the 2-methyl-3-oxopentanoic acid thioester intermediate. In fact, introduction of the loop from DEBS module 1 at AvrII-HpaI sites (pJCE1; sites used successfully for both DEBS module 2 and DEBS module 5) gave a chimaeric multienzyme whose products were traces only of the ketolactones **2a** and **2b** (Table 2), with none of the expected hydroxylactones **4a** and **4b**.

If the expected diastereoisomeric alcohol had been formed, it would have been released from the multienzyme, since the DEBS thioesterase (TE) does not discriminate significantly in rates of hydrolysis of diastereomeric thioester substrates differing in configuration at C-2 and/or C-3.[49] This result can be explained (Scheme 1) if the KR_1 domain is confronted in the chimaeric module 2 by the unepimerised (2*S*)-2-methyl-3-oxopentanoic acid thioester intermediate, which it is unable to reduce directly; this is in agreement with our previous in vitro experiments with purified recombinant KR_1 enzyme.[42]

Recent speculation based on modelling of substrates into an unliganded X-ray crystal structure of the tylosin KR1 domain has led to the proposal[9] that KR_1 is itself capable of being an active agent of epimerization, a possibility raised independently by other authors based on bioinformatic analysis.[50] The present results do not support this hypothesis. Independently, others have recently shown that recombinant KR_1 does not reduce or epimerise (2*R*)-2-methyl-3-oxopentanoyl-ACP acid generated by recombinant KS_3-AT_3.[51] Conceivably, KR_1 is inactive in the chimaeric module for reasons connected for example with specific unfavourable protein-protein interactions. However, when the RIFS module 8 loop was introduced at SnaBI-NheI sites (which worked well for RIFS module 7) a significant amount (27 mg L^{-1}) of the ketolactones **3a** and **3b**, together with, again, only traces of the (2*S*,3*S*)-hydroxylactones **5a** and **5b** were produced. This would have been expected if the RIFS module 8 KR had behaved as it does in its native context (Scheme 1). Again, it would appear that the incoming KR is presented with an epimer of the ketoacyl-ACP substrate which it can neither reduce directly, nor epimerise to an isomer that it could then reduce.



Scheme 1. Analysis of the stereochemistry of ketoreduction and the role of epimerisation. Lactones **1a** and **3a** are derived from the (2*R*)-2-methyl-3-ketoacyl-ACP intermediate generated in DEBS1-TE module 2 through reduction on opposite faces of the 3-keto group. Lactones **4a** and **5a** are the hypothetical products from hybrid DEBS1-TE containing either DEBS KR_1 or RIFS KR_7 in the reductive loop of module 2 based on the activity of these reductive enzymes in their native context, but are not expected to form owing to the absence of catalysed epimerisation activity in DEBS module 2.[26,27,42]

Conclusions

The systematic variation allowed by the polylinker approach for swapping reductive loops in modular PKSs has shown both the robustness of the domain-swap technology for creating functional hybrid PKSs, and also some of its limitations. Encouragingly, it has proved possible to find a combination of donor loop and splice sites that allow most of the desired products to be obtained in reasonable or even very good yield. The obvious exceptions were certain products altered in their configuration at C-2 (methyl) and C-3 (hydroxy), but even here it has been possible to rationalise the pattern of products obtained on the basis of what is already known about the molecular basis of Celmer's rules—that the stereochemical outcome of polyketide chain extension on modular PKSs depends crucially on the dynamic interplay between KS and KR domains. Further work is still needed, however, to locate the seat of the epimerisation activity within epimerising modules, and it may even be that such activity normally demands collaboration of both KS and KR partners within an intact PKS module, whether as spectators or active agents. Meanwhile, the polylinker approach

has shown the importance from a synthetic viewpoint of trying more than one example of a desired hybrid. The results found here illustrate clearly that a different choice of splice sites, and/or use of a different donor reductive loop, can very significantly affect the yield of the desired novel polyketide. No single donor and no single pair of splice sites were found to be reliably optimal to effect a given alteration. This more pragmatic approach has recently been independently adopted with success in piecing together whole PKS modules that were created from synthetic DNA for better expression in *E. coli*.[52] As the price of synthetic genes drops and the number of sequenced natural PKS modules grows, such flexible strategies will become increasingly appealing.

Reductive loop swapping may owe its success at least in part to fundamental features of PKS structure and evolution. Dittmann and colleagues[10] have recently conducted a thoughtful analysis of the reconstructed evolutionary relationships of different domain types within the modular PKSs of *Streptomyces avermitilis*. Their phylogenetic analysis suggests that the majority of modules in the various PKSs of this organism have been altered over time by recombination within and between PKS-containing biosynthetic gene clusters. In particular, it appears that this "natural reprogramming"[10] is never observed for KS domains, but only for AT, KR and DH-KR replacements. The hotspots for natural PKS recombination that they highlight in their analysis include precisely those regions (respectively C-terminal of the AT domain, and N-terminal of the ACP domain) that are common to all modules and that we have used in the present study as the preferred splice points for reductive loop swapping. Other recent evidence also supports the view that tactics adopted in the laboratory over the last few years to engineer modular PKS are fully precedented in natural recombination, including AT domain swaps[53] and whole-module deletions.[54]

Experimental Section

General methods: *Saccharopolyspora erythraea* JC2 (Δ*eryA*) NCIMB 40802 and its cultivation have been previously described.[23,32] Standard procedures for DNA isolation and manipulation were performed as described previously.[55,56] Isolation of DNA fragments from agarose gel and purification of PCR products was carried out using the Nucleo-Spin 2-in-1 Extract kit (Macherey–Nagel, Düren, Germany). Standard PCR reactions were performed with *Pfu* polymerase (Stratagene); reactions were performed on a programmable RoboCycler Gradient 96 (Stratagene). Synthetic oligonucleotides were purchased from VH Bio (Gateshead, UK), and automated DNA sequencing was carried out with double-stranded templates by using an automated ABI Prism 3700 DNA analyser (Applied Biosystems). Protoplast transformation of *S. erythraea* with plasmid DNA has been previously described.[32]

Construction of expression constructs for DEBS1-TE containing a polypeptide linker in place of the KR_2 domain: Plasmid pJLK114 is a plasmid based on pCJR24[32] that contains a truncated erythromycin PKS (DEBS) gene comprising the loading module, the first and second extension modules and the chain-terminating thioesterase, except that the DNA segment encoding the KR domain from module 2 has been substituted by a synthetic AvrII-HpaI oligonucleotide linker containing unique recognition sites for six other restriction enzymes (Figure 1C). It was constructed through several intermediate plasmids as detailed in the Supporting Information. A second version of this expression plasmid for DEBS1-TE, containing an NheI site in place of the HpaI site, was also constructed, as detailed in the Supporting Information.

Construction of DEBS1-TE expression plasmids containing altered reductive loops: Each targeted set of reductive domains ("reductive loops") was amplified from cosmid templates using appropriate pairs of synthetic mutagenic oligonucleotide primers to introduce flanking restriction sites, allowing direct cloning into either plasmid pJLK114 or pJLK117. Four different reductive loops were selected from the erythromycin PKS (DEBS): KR_2 as a SpeI-NsiI fragment, KR_1 as an AvrII-HpaI fragment, DH_4-ER_4-KR_4 as a BglII-Bsu36I fragment, and KR_5 as an AvrII-HpaI fragment. Four reductive loops were selected from the rapamycin PKS: DH_1-ER_1-KR_1 as a BglII-NheI fragment, DH_4-KR_4 as an AvrII-HpaI fragment, DH_{10}-KR_{10} as a BglII-NheI fragment, and DH_{13}-ER_{13}-KR_{13} as an AvrII-HpaI fragment. From the avermectin PKS, KR_1 was amplified both as a BglII-NheI fragment and as a SnaBI-Bsu36I fragment. The KR_1 of the tylosin PKS was amplified as a BglII-NheI fragment. From the rifamycin PKS, KR_7 and KR_8 were each amplified as a SnaBI-NheI fragment. All inserts were checked by DNA sequencing.

Plasmid pJLK25 is a pJLK114-based plasmid in which a DNA fragment encoding the reductive loop of the second module of the erythromycin PKS (the KR_2 domain) was amplified using oligonucleotides oLK25F and oLK25R (Table S1) and cloned into pUC18, before being ligated into the multiple cloning site at SpeI and NsiI sites.

Analysis of triketide fermentation products: Extraction of triketides from lawns of recombinant *S. erythraea* grown on SM3 or TWM agar plates was done by the "lactonex" procedure: a 1 cm^2 piece of mycelium and agar was cut out, chopped into pieces, and transferred into a 2 mL Eppendorf tube. Ethyl acetate (1.2 mL) and formic acid (20 μL) were added to each tube with mixing. The tubes were incubated at 50 °C for 15 min, and then vortexed for 30 min. The mixture was centrifuged for 1 min and the supernatant removed carefully and placed in a fresh 1.5 mL Eppendorf tube. After evaporation to dryness under reduced pressure, the residue was dissolved in 50 μL of ethyl acetate, centrifuged for 1 min and the supernatant was transferred to a GC-MS vial for analysis. For cultures grown for 6 days in SM3 liquid medium, 1 mL of clarified supernatant was extracted first with of ethyl acetate (0.9 mL), and then again with ethyl acetate (0.5 mL), and the combined organic extracts were evaporated under reduced pressure.

For analysis of 2-enoic acid triketides, dried samples were dissolved in of acetone (600 μL) and treated with diazomethane (generated from Diazald, Aldrich) until the yellow colour persisted. The Eppendorf tubes were then initially left in a fume cupboard to allow evaporation, and then briefly dried in a speedvac. The samples were then dissolved in ethyl acetate (50 μL) for analysis by GC-MS. GC-MS analysis was carried out on a Thermo Finnigan GCQ instrument in CI mode on a Phenomenex AB-5 column (30 m × 25 mm × 25 μm) with the following temperature gradient: 40 °C for 2 min; 10 °C min^{-1} to 250 °C; 25 °C min^{-1} to 300 °C.

Supernatants were spiked with one or more synthetic standard triketide lactones both to confirm the assignment of peaks to particular diastereoisomers (as exemplified in Figures 4 and 5). The yields given in Tables 1 and 2 represent unoptimised total amounts (**a** plus **b** forms) of each triketide, from individual experiments after growth on SM3 agar plates, unless detailed otherwise. Yields were

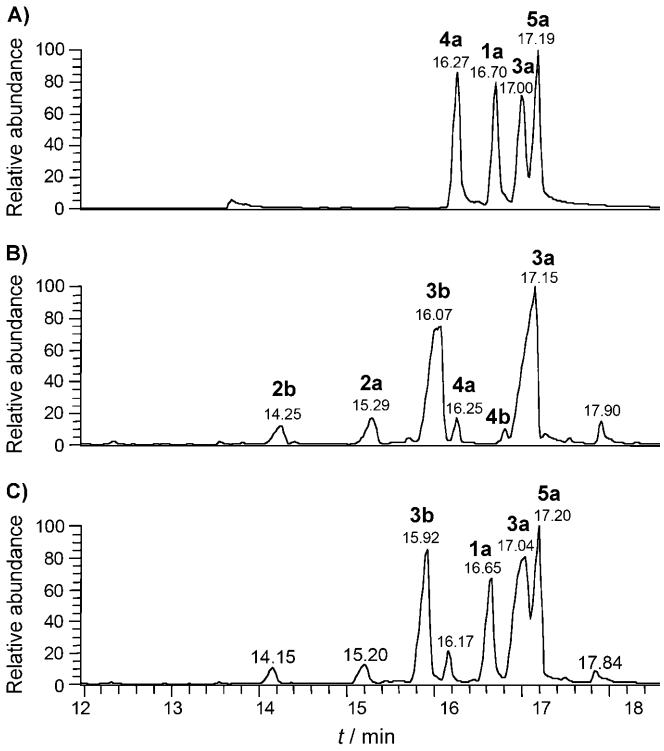


Figure 4. GC-MS separation and identification of triketide lactones from fermentation of A) *S. erythraea* JC2(pJLK35), containing a hybrid DEBS1-TE housing a reductive loop derived from tylosin PKS (TYLS) module 1. B) Separation of standard 3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lactones **1a**, **3a**, **4a** and **5a**. C) Triketide lactones from JC2(pJLK35). To aid in peak assignment, samples were spiked with authentic standard samples of individual diastereomers of the expected 3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lactones **1a** and **5a**. For further details, see Figure S2.

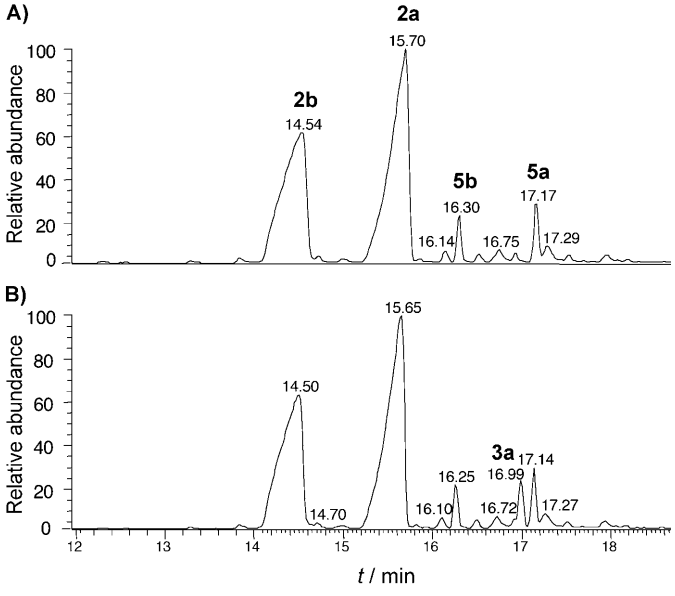


Figure 5. GC-MS separation and identification of triketide lactones from fermentation of A) *S. erythraea* JC2 (pRIF7), containing a hybrid DEBS 1-TE housing a reductive loop derived from rifamycin PKS module 7. B) Triketide lactones from JC2 (pRIF7). To aid in peak assignment, samples were spiked with a synthetic sample of one of the diastereoisomers **3a** of the expected 3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lactones. For further details, see Figure S3.

estimated by admixture of supernatants with known amounts of synthetic reference triketide lactones and measurement of relative peak areas after GC separation. Experiments were repeated 2–3 times, with estimated yields ranging 10–30% either side of the values quoted in Tables 1 and 2.

Acknowledgements

We thank Dr Hui Hong and Dr Kira Weissman for advice on the preparation of this paper. Support for this work was provided by the BBSRC (U.K.) through project grants to P.F.L. and J.S., and a postgraduate studentship to I.S.G. L.K. was a Postdoctoral Research Fellow supported by the Schweizerische Nationalfonds.

Keywords: erythromycin · natural products · polyketides · *Streptomyces* · synthetic biology

[1] J. Staunton, K. J. Weissman, *Nat. Prod. Rep.* **2001**, *18*, 380–416.
[2] C. Khosla, Y. Tang, A. Y. Chen, N. A. Schnarr, D. E. Cane, *Annu. Rev. Biochem.* **2007**, *76*, 195–221.
[3] K. J. Weissman, R. Müller, *ChemBioChem* **2008**, *9*, 826–848.
[4] S. Smith, S.-C. Tsai, *Nat. Prod. Rep.* **2007**, *24*, 1041–1072.
[5] J. F. Aparicio, P. Caffrey, A. F. A. Marsden, J. Staunton, P. F. Leadlay, *J. Biol. Chem.* **1994**, *269*, 8524–8528.
[6] J. Staunton, P. Caffrey, J. F. Aparicio, G. A. Roberts, S. S. Bethell, P. F. Leadlay, *Nat. Struct. Biol.* **1996**, *3*, 188–192.
[7] A. T. Keatinge-Clay, R. M. Stroud, *Structure* **2006**, *14*, 737–748.
[8] C. Richter, D. A. Stanmore, R. N. Miguel, M. C. Moncrieffe, L. Tran, S. Brewerton, F. Meersman, R. W. Broadhurst, K. J. Weissman, *FEBS J.* **2007**, *274*, 2196–2209.
[9] A. T. Keatinge-Clay, *Chem. Biol.* **2007**, *14*, 898–908.
[10] H. Jenke-Kodama, T. Börner, E. Dittmann, *PLoS Comput. Biol.* **2006**, *2*, e132.
[11] S. Donadio, M. J. Staver, J. B. McAlpine, L. Katz, *Science* **1991**, *252*, 675–679.
[12] S. Donadio, J. B. McAlpine, P. J. Sheldon, M. Jackson, L. Katz, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 7119–7123.
[13] D. Bedford, J. R. Jacobsen, G. Luo, D. E. Cane, C. Khosla, *Chem. Biol.* **1996**, *3*, 827–831.
[14] M. Oliynyk, M. J. Brown, J. Cortés, J. Staunton, P. F. Leadlay, *Chem. Biol.* **1996**, *3*, 833–839.
[15] S. Kuhstoss, M. Huber, J. R. Turner, J. W. Paschal, R. N. Rao, *Gene* **1996**, *183*, 231–236.
[16] S. Gaisser, J. L. Kellenberger, A. Kaja, A. J. Weston, R. E. Lill, S. G. Kendrew, L. Low, R. M. Sheridan, B. Wilkinson, I. S. Galloway, K. Stutzman-Engwall, H. A. I. McArthur, J. Staunton, P. F. Leadlay, *Org. Biomol. Chem.* **2003**, *1*, 2840–2847.
[17] C. M. Kao, M. McPherson, R. McDaniel, H. Fu, D. E. Cane, C. Khosla, *J. Am. Chem. Soc.* **1997**, *119*, 11339–11340.
[18] C. M. Kao, M. McPherson, R. McDaniel, H. Fu, D. E. Cane, C. Khosla, *J. Am. Chem. Soc.* **1998**, *120*, 2478–2479.
[19] Y. J. Yoon, B. J. Beck, B. S. Kim, H.-Y. Kang, K. A. Reynolds, D. H. Sherman, *Chem. Biol.* **2002**, *9*, 203–214.
[20] R. McDaniel, C. M. Kao, S. J. Hwang, C. Khosla, *Chem. Biol.* **1997**, *4*, 667–674.
[21] R. McDaniel, A. Thamchaipenet, C. Gustafsson, H. Fu, M. Betlach, M. Betlach, G. Ashley, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1846–1851.
[22] W. D. Celmer, *J. Am. Chem. Soc.* **1965**, *87*, 1801–1802.
[23] J. Cortés, K. E. Wiesmann, G. A. Roberts, M. J. Brown, J. Staunton, P. F. Leadlay, *Science* **1995**, *268*, 1487–1489.
[24] A. F. A. Marsden, P. Caffrey, J. F. Aparicio, M. S. Loughran, J. Staunton, P. F. Leadlay, *Science* **1994**, *263*, 378–380.
[25] K. E. H. Wiesmann, J. Cortés, M. J. Brown, A. L. Cutter, J. Staunton, P. F. Leadlay, *Chem. Biol.* **1995**, *2*, 583–589.
[26] K. J. Weissman, M. Timoney, M. Bycroft, P. Grice, U. Hanefeld, J. Staunton, P. F. Leadlay, *Biochemistry* **1997**, *36*, 13849–13855.

[27] I. Böhm, I. E. Holzbaur, U. Hanefeld, J. Cortés, J. Staunton, P. F. Leadlay, *Chem. Biol.* **1998**, *5*, 407–412.
[28] I. E. Holzbaur, R. C. Harris, M. Bycroft, J. Cortés, C. Bisang, J. Staunton, B. A. M. Rudd, P. F. Leadlay, *Chem. Biol.* **1999**, *6*, 189–195.
[29] L. H. Østergaard, L. Kellenberger, J. Cortés, M. P. Roddis, M. Deacon, J. Staunton, P. F. Leadlay, *Biochemistry* **2002**, *41*, 2719–2726.
[30] I. E. Holzbaur, A. Ranganathan, I. P. Thomas, D. J. A. Kearney, J. A. Reather, B. A. M. Rudd, J. Staunton, P. F. Leadlay, *Chem. Biol.* **2001**, *8*, 329–340.
[31] H. Hong, A. N. Appleyard, A. P. Siskos, J. Garcia-Bernardo, J. Staunton, P. F. Leadlay, *FEBS J.* **2005**, *272*, 2373–2387.
[32] C. J. Rowe, J. Cortés, S. Gaisser, J. Staunton, P. F. Leadlay, *Gene* **1998**, *216*, 215–223.
[33] A. L. Wilkinson, U. Hanefeld, B. Wilkinson, P. F. Leadlay, J. Staunton, *Tetrahedron Lett.* **1998**, *39*, 9827–9830.
[34] A. Ranganathan, M. Timoney, M. Bycroft, J. Cortés, I. P. Thomas, B. Wilkinson, J. L. Kellenberger, U. Hanefeld, I. S. Galloway, J. Staunton, P. F. Leadlay, *Chem. Biol.* **1999**, *6*, 731–741.
[35] U. Hanefeld, A. M. Hooper, J. Staunton, *Synthesis* **1999**, 401–403.
[36] Z. A. Hughes-Thomas, C. B. Stark, I. U. Böhm, J. Staunton, P. F. Leadlay, *Angew. Chem.* **2003**, *115*, 4613–4616; *Angew. Chem. Int. Ed.* **2003**, *42*, 4475–4478.
[37] R. McDaniel, C. M. Kao, H. Fu, P. Hevezi, C. Gustafsson, M. Betlach, G. Ashley, D. E. Cane, C. Khosla, *J. Am. Chem. Soc.* **1997**, *119*, 4309–4310.
[38] P. Caffrey, *ChemBioChem* **2003**, *4*, 654–657.
[39] R. Reid, M. Piagentini, E. Rodriguez, G. Ashley, N. Viswanathan, J. Carney, D. V. Santi, C. R. Hutchinson, R. McDaniel, *Biochemistry* **2003**, *42*, 72–79.
[40] B. J. Rawlings, *Nat. Prod. Rep.* **1997**, *14*, 335–358.
[41] J. Wu, T. J. Zaleski, C. Valenzano, C. Khosla, D. E. Cane, *J. Am. Chem. Soc.* **2005**, *127*, 17393–17404.
[42] A. P. Siskos, A. Baerga-Ortiz, S. Bali, V. Stein, H. Mandami, D. Spiteller, B. Popovic, J. B. Spencer, J. Staunton, K. J. Weissman, P. F. Leadlay, *Chem. Biol.* **2005**, *12*, 1145–1153.
[43] A. Baerga-Ortiz, B. Popovic, A. P. Siskos, H. M. O'Hare, D. Spiteller, M. G. Williams, N. Campillo, J. B. Spencer, P. F. Leadlay, *Chem. Biol.* **2006**, *13*, 277–285.
[44] H. M. O'Hare, A. Baerga-Ortiz, B. Popovic, J. B. Spencer, P. F. Leadlay, *Chem. Biol.* **2006**, *13*, 287–296.
[45] E. Cundliffe, N. Bate, A. Butler, S. Fish, A. Gandecha, A. , L. Merson-Davies, *Antonie Van Leeuwenhoek* **2001**, *79*, 229–234.
[46] D. J. MacNeil, J. L. Occi, K. M. Gewain, T. MacNeil, P. H. Gibbons, C. L. Ruby, S. J. Danis, *Gene* **1992**, *115*, 119–125.
[47] H. Ikeda, T. Nonomiya, M. Usami, T. Ohta, S. Ōmura, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9509–9514.
[48] L. Tang, Y. J. Yoon, C. Y. Choi, C. R. Hutchinson, *Gene* **1998**, *216*, 255–265.
[49] K. J. Weissman, C. J. Smith, U. Hanefeld, R. Aggarwal, M. Bycroft, J. Staunton, P. F. Leadlay, *Angew. Chem.* **1998**, *110*, 1503–1506; *Angew. Chem. Int. Ed.* **1998**, *37*, 1437–1439.
[50] A. Starcevic, M. Jaspers, J. Cullum, D. Hranueli, P. F. Long, *ChemBioChem* **2007**, *8*, 28–31.
[51] R. Castonguay, W. He, A. Y. Chen, C. Khosla, D. E. Cane, *J. Am. Chem. Soc.* **2007**, *129*, 13758–13769.
[52] H. G. Menzella, R. Reid, J. R. Carney, S. S. Chandran, S. J. Reisinger, K. G. Patel, D. A. Hopwood, D. V. Santi, *Nat. Biotechnol.* **2005**, *23*, 1171–1176.
[53] H. Hong, J. B. Spencer, J. L. Porter, P. F. Leadlay, T. Stinear, *ChemBioChem* **2005**, *6*, 643–648.
[54] H. Hong, T. Stinear, P. Skelton, J. B. Spencer, *J. C. S. Chem. Commun.* **2005**, 4306–4308.
[55] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, New York, **2000**.
[56] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, D. A. Hopwood, *Practical Streptomyces Genetics*, 2nd ed., John Innes Foundation, Norwich, **2000**.

Received: May 15, 2008
Published online on October 20, 2008

