A Mutant Generated by Expression of an Engineered DEBS1 Protein from the Erythromycin-producing Polyketide Synthase (PKS) in *Streptomyces coelicolor* Produces the Triketide as a Lactone, but the Major Product is the nor-Analogue Derived from Acetate as Starter Acid

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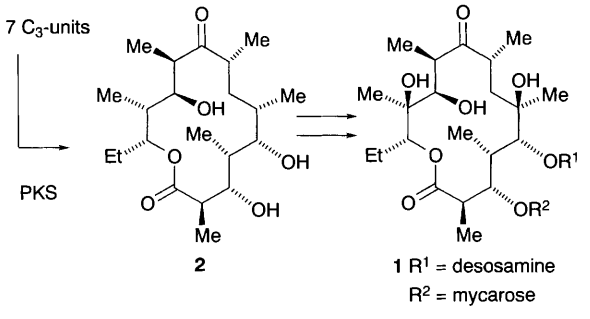
Genetic engineering of theerythromycin polyketide synthase (PKS), to generate the DEBS 1 protein with a fused thioesterase domain (DEBS 1-TE), followed by expression in *Streptomyces coelicolor*, resulted in a mutant which produces a small quantity of the erythromycin triketide as a lactone, but the major product is the corresponding nor-analogue derived from acetate rather than propionate as the starter acid.

The simplest biosynthetic intermediate on the pathway to erythromycin A **1** to be isolated from blocked mutants is 6-deoxyerythronolide B **2**.[1] It was long suspected that this was generated (Scheme 1) by condensation of seven C_3-units on a polyketide synthase (PKS) but prolonged efforts to identify the responsible proteins and intermediates proved fruitless.

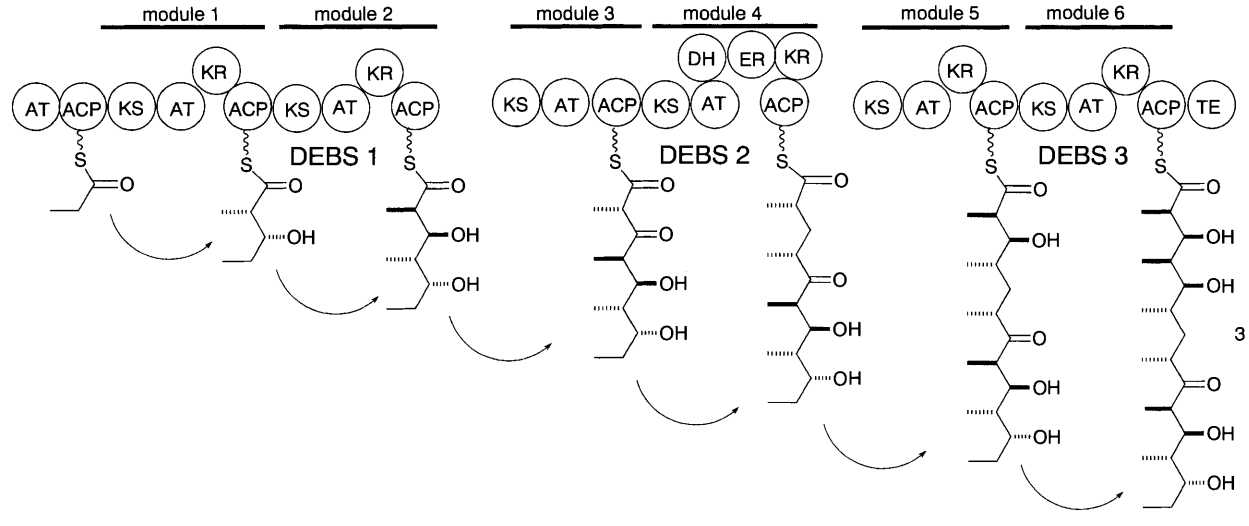
The first breakthrough came in 1990 with the identification of one of the genes coding for the PKS.[1] The corresponding protein was exceptionally large (M_r 330 kDa) and sequence homology with known fatty acid synthase enzymes supported the suggestion that it folded to form nine independent but structurally linked globular domains. The predicted catalytic activities of the domains were sufficient for two independent cycles of polyketide chain extension followed by cleavage of the thioester link to release the product. Subsequently, two other similar giant proteins were also implicated, establishing that the PKS as a whole is a remarkably large structure with a separate catalytic site for each step of chain assembly leading ultimately to the heptaketide intermediate **3** (Scheme 2).[1] Finally, the thioester link to **3** is cleaved by a strategically placed thioesterase domain (TE) to produce the macrocyclic lactone **2**.



Scheme 1 Overview of the Erythromycin biosynthetic pathway

The ordering of the domains follows a pattern which suggests that they might be organised spatially into six independent modules, one for each chain extension cycle. Support for this idea was provided by gene disruption experiments which were designed to produce mutant organisms containing modified proteins in which the catalytic activity of key domains was selectively destroyed. Analogues of the normal metabolites were produced in two of these mutants.[2]

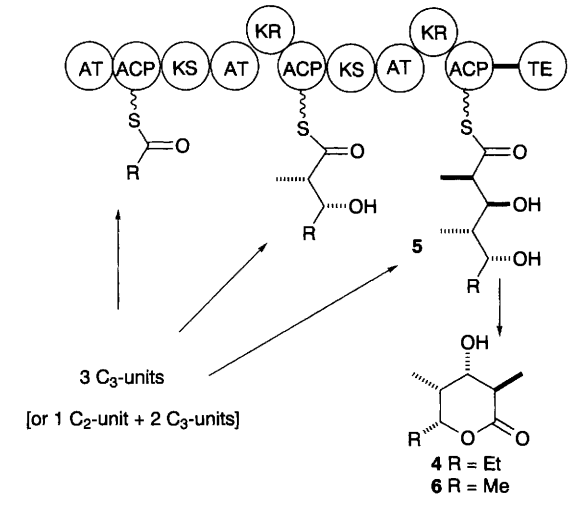
A more challenging, but a potentially much more versatile strategy for genetic engineering, is to reposition catalytic domains rather than destroy them. If the outcome of the overall chain extension process is indeed dictated by the ordering of the catalytic domains, it may be possible to produce a wide range of novel polyketide natural products in a predictable way. We recently reported the first successful experiment of this kind in which the erythromycin PKS was modified by moving a thioesterase domain from the end of DEBS 3 to the end of DEBS 1.[3] The mutant PKS, DEBS 1-TE, was expressed in the normal host organism *Saccharospora erythraea* and it led to the efficient production of the predicted product, the δ-lactone **4** (Scheme 3). In its new context, therefore, the repositioned TE domain proved to be highly effective at cleaving the thioester link at the neighbouring acyl carrier protein (ACP), even though the structure of the acyl chain of the triketide **5** was very different from its normal substrate, the heptaketide **3**. The metabolite was produced by this mutant in a yield comparable with that of the normal metabolite erythromycin and there was no detectable competing formation of diastereoisomers or homologues.



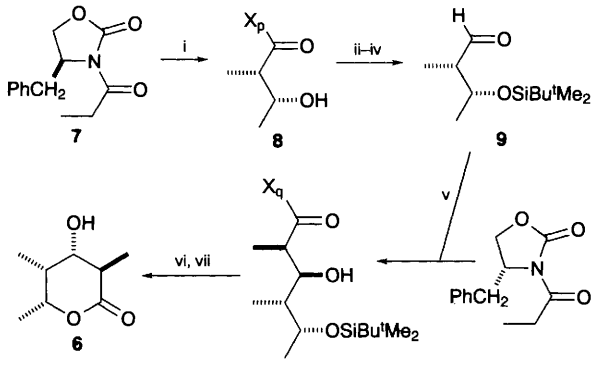
Scheme 2 The Erythromycin PKS: relating proteins (DEBS), modules and biosynthetic steps

In view of these results we were intrigued by a recent report of a superficially similar experiment at Stanford in which the unmodified DEBS 1 was expressed on its own in a foreign host, *Streptomyces coelicolor*.[4] The only product reported was the triketide lactone **3**. In contrast, a mixture of products was obtained earlier in a closely related Stanford experiment in which all three DEBS proteins were expressed in the same host: the product mixture contained the expected product **2** and, in addition, the nor-analogue produced by use of acetate as starter acid rather than propionate.[5] This mixed metabolism probably reflects an inadequacy of the host organism rather than the use of the unmodified DEBS 1 protein, in that the feeder pathways which generate biosynthetic building blocks in *S. coelicolor* are geared to the production of polyacetate metabolites rather than polypropionates.

In view of the two Stanford experiments we have investigated the behaviour of the genetically engineered Cambridge protein, DEBS 1-TE, in *S. coelicolor*. The resulting mutant grew satisfactorily but analysis of the organic extracts by TLC showed that the normal triketide lactone **4** was only a minor component. The major DEBS product was isolated and identified as the corresponding nor-compound **6** formed by use of acetate as starter acid rather than propionate. The mass spectrum was consistent with a structure having one fewer methylene residues. The signals in the NMR spectrum of the novel compound were broadly similar to equivalent signals of **4** with the exception of those from the starter residue, which appeared at δ 1.34 (3 H, d, *J* 6.6 Hz) and 4.43 (1 H, qd, *J* 6.6 and 2.5 Hz).



Scheme 3



Scheme 4 *Reagents and conditions*: i, $Bu_2BOSO_2CF_3$, Et_3N, MeCHO, H_2O_2; ii, $AlMe_3$, MeONHMe·HCl; iii, Bu^tMe_2SiCl, imidazole, DMAP; iv, DIBAL-H; v, $Bu_2BOSO_2CF_3$, Et_3N; vi, H_2O_2, LiOH; vii, HCl, THF

An authentic sample of this metabolite was synthesised by the route shown in Scheme 4. Condensation of the propionate derivative **7** of the Evans' chiral auxiliary with acetaldehyde using boron enolate methodology[6] gave the diketide analogue **8**. This was converted to the aldehyde **9** and the hydroxy group protected. The chain was then further extended using Evans' methodology but with the enantiomer of **7** as the chiral auxiliary. The resulting product was cyclised and deprotected to give the lactone **6**. The synthetic and natural products were spectroscopically (IR, ^1H NMR) and chromatographically (TLC and HPLC) identical.

The engineered construct DEBS 1-TE has now been expressed in two hosts, the natural host,[3] *S. erythraea*, and an unnatural host, *S. coelicolor*. The total yields of products from the polyketide synthase were similar, but in the first host a single product was obtained whereas in the second a mixture of products resulted. Clearly the environment of the host organism can profoundly influence the performance of a polyketide synthase.

A second important conclusion can be drawn from comparison of the present results with DEBS 1-TE in *S. coelicolor* with the earlier work[4] with the isolated DEBS 1 in the same host. DEBS 1-TE is rationally engineered to have an in-built mechanism for relase of its product. The advantage of this strategy is strikingly demonstrated by the significantly increased yield of the polyketide products, confirming our equivalent results[3] in *S. erythraea*. Regrettably, the earlier report[4] did not address the obvious possibility that an alternative product **6**, formed by the isolated DEBS 1 using acetate as starter unit, might be present in the crude extract from the host organism. It is therefore not safe to make comparisons of product specificity of the two constructs in these experiments.

Finally, our results with the DEBS 1-TE show that the TE of the erythromycin PKS can function out of its natural context with different substrates. This novel strategy for engineering polyketide systems may be more widely applicable, not just in the erythromycin PKS but in other PKS systems. In the following paper we show that the TE has the requisite broad substrate specificity.

We thank the SERC and Ciba-Geigy plc for financial support.

Received, 24th March 1995; Com. 5/01884F

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