Polyketide synthesis *in vitro* on a modular polyketide synthase

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Background: The 6-deoxyerythronolide B synthase (DEBS) of *Saccharopolyspora erythraea*, which synthesizes the aglycone core of the antibiotic erythromycin A, contains some 30 active sites distributed between three multienzyme polypeptides (designated DEBS1–3). This complexity has hitherto frustrated mechanistic analysis of such enzymes. We previously produced a mutant strain of *S. erythraea* in which the chain-terminating cyclase domain (TE) is fused to the carboxyl-terminus of DEBS1, the multienzyme that catalyzes the first two rounds of polyketide chain extension in *S. erythraea*. This mutant strain produces triketide lactone *in vivo*. We set out to purify the chimaeric enzyme and to determine its activity *in vitro*.

Results: The purified DEBS1–TE multienzyme catalyzes synthesis of triketide lactones *in vitro*. The synthase specifically uses the (2*S*)-isomer of methylmalonyl-CoA, as previously proposed, but has a more relaxed specificity for the starter unit than *in vivo*.

Conclusions: We have obtained a purified polyketide synthase system, derived from DEBS, which retains catalytic activity. This approach opens the way for mechanistic and structural analyses of active multienzymes derived from any modular polyketide synthase.

Chemistry & Biology September 1995, 2:583–589

Key words: cyclase, erythromycin, polyketide synthase, *Saccharopolyspora erythraea*, triketide lactone

Introduction

Complex polyketides are natural products, found predominantly in *Streptomyces* and related filamentous bacteria, that exhibit an impressive range of antibiotic, anticancer, antiparasite and immunosuppressant activities. Despite their apparent structural diversity, they are synthesized by a common pathway in which units derived from acetate or propionate are condensed onto the growing chain in a process resembling fatty acid biosynthesis [1,2]. The intermediates remain bound to the polyketide synthase (PKS) during multiple cycles of chain extension and (to a variable extent) reduction of the β-keto group formed in each condensation. The structural variation between naturally occurring polyketides arises largely from the way in which each PKS controls the number and type of units added, and from the extent and stereochemistry of reduction at each cycle. Still greater diversity is produced by the action of regiospecific glycosylases, methyltransferases and oxidative enzymes on the product of the PKS.

Complex reduced polyketides, such as the macrolides and polyethers, are synthesized in bacteria on multifunctional or type I PKSs that contain a separate set, or module, of enzymatic activities for every round of chain extension [3–6]. For example, the clinically-important antibiotic erythromycin A is derived from propionyl-CoA and six molecules of (2*S*)-methylmalonyl-CoA [7], through the sequential action of six such modules housed in the three multienzyme polypeptides [8] of 6-deoxyerythronolide B synthase (DEBS) (Fig. 1). The most remarkable example studied thus far is the PKS for the immunosuppressant rapamycin from *Streptomyces hygroscopicus*, which contains 14 modules contained within only three huge multienzyme polypeptides [9].

Progress in understanding the enzymology of such modular type I systems has been frustrated by the lack of a cell-free system to study polyketide chain synthesis by any of these multienzymes, although several partial reactions of DEBS have been successfully assayed *in vitro* [7,10,11]. We recently introduced [12] a novel, efficient and potentially general method for the controlled early release of polyketide chains from a modular type I PKS, by relocation of the cyclase/thioesterase (TE) domain from DEBS3, which normally catalyzes the fifth and sixth rounds of chain extension, to the carboxyl-terminus of DEBS1, which catalyzes the first two rounds (Fig. 1). Mutants of *S. erythraea* [12] or of *S. coelicolor* [13] that lack endogenous DEBS genes but express the chimaeric DEBS1–TE fusion protein were shown to produce the expected triketide lactone **1B** (Fig. 1) in good yield, together with variable amounts of a second triketide lactone **1A**.

We show here that cell extracts from such a strain are also able to synthesize triketide lactones **1A** and **1B**, in the presence of methylmalonyl-CoA, an acyl-CoA

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 ISSN 1074-5521

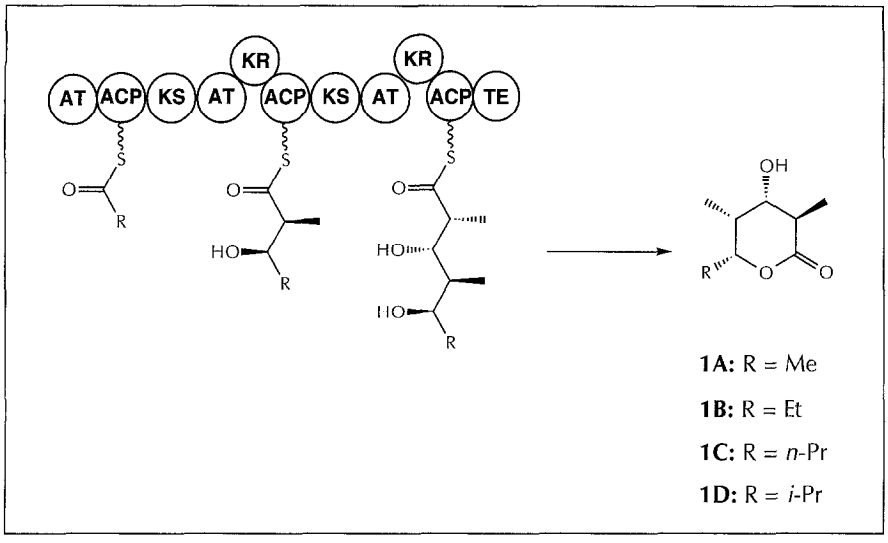

Fig. 1. The domain organization of the 'triketide lactone synthase' (DEBS1–TE). Each protein module contains an acyl carrier protein (ACP), an acyltransferase (AT), a β-ketoacyl-ACP synthase (KS) and β-ketoacyl-ACP reductase (KR). The thioesterase-cyclase (TE) releases the triketide lactone product (normally compound **1B**) from the synthase [12].

(such as propionyl-CoA), and NADPH. Furthermore, DEBS1–TE has been purified from the extracts to homogeneity and retains its activity. We report here the use of this *in vitro* system to explore several important aspects of the specificity and stereospecificity of a typical modular polyketide synthase.

Results and discussion

Demonstration of cell-free polyketide biosynthesis

Cell extracts were prepared from the DEBS1–TE-expressing *S. erythraea* strain JCB101 as described in Materials and methods, and used immediately in experiments to detect the conversion of propionyl-CoA and methylmalonyl-CoA into triketide lactone **1B**. Radioactivity was added in the form of either [^{14}C]-propionyl-CoA or [^{14}C]-(*R,S*)-methylmalonyl-CoA, or both, and the reaction mixtures were quenched by extraction with ethyl acetate. The radioactive products were separated either by thin-layer chromatography (TLC), or by normal-phase or reverse-phase high-pressure liquid chromatography (HPLC), and their relative mobilities or elution times were compared with those of authentic [^{14}C]-triketide lactone **1B**.

A typical profile for the production of radiolabelled compound **1B** over time is shown in Fig. 2, in an experiment in which the extract was incubated with propionyl-CoA, (*R,S*)-methylmalonyl-CoA, and NADPH. Purified methylmalonyl-CoA epimerase from *Propionibacterium shermanii* [14] was added to ensure rapid equilibration of the two stereoisomers, as only (2*S*)-methylmalonyl-CoA is apparently used by DEBS [7].

Although the concentrations of propionyl-CoA and methylmalonyl-CoA used here are unlikely to be optimal, this simple *in vitro* system has allowed several

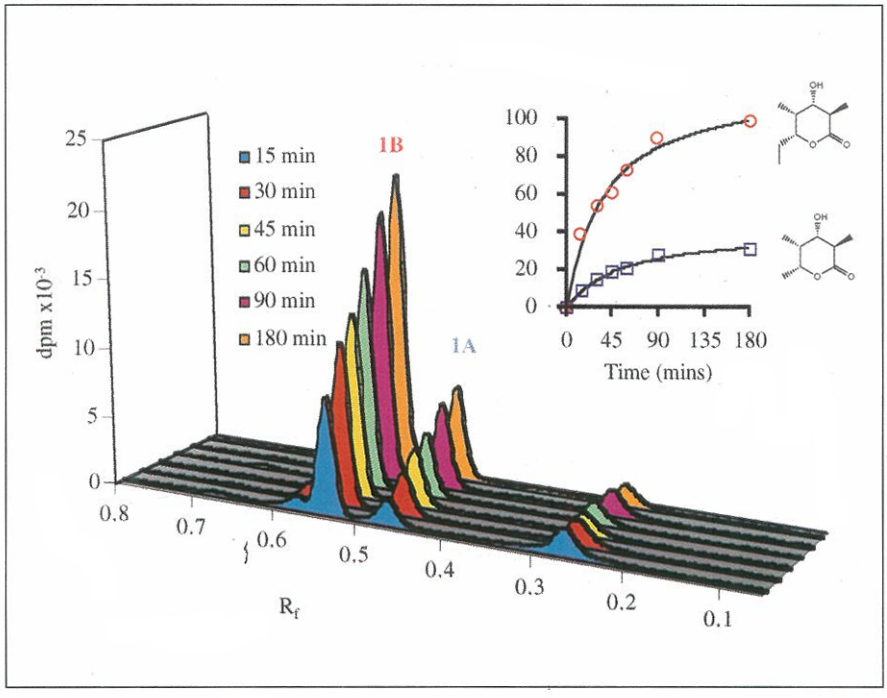

Fig. 2. Cell extracts containing DEBS1–TE generate triketide lactones *in vitro*. The figure shows production of ^{14}C-labelled triketide lactones **1A** and **1B** from ^{14}C-labelled 300 μM propionyl-CoA, 600 μM (27nCi) (*RS*)-methylmalonyl-CoA, and 600 μM NADPH in the presence of methylmalonyl-CoA epimerase in cell extracts of *S. erythraea* at different time points. The lactones were separated on silica TLC plates and radioactivity was detected by two-dimensional phosphorimaging. Lactone **1A** has R_f 0.58, **1B** has R_f 0.47. The inset summarizes the increase in production of **1A** (lower curve) and **1B** (upper curve) with time. Production is measured in arbitrary units. The material at R_f 0.28 is unidentified. For details see the text.

other features of polyketide chain extension to be examined, for example, by using well known inhibitors of specific enzymatic activities. We previously showed [14] that the active site serine of DEBS1–TE, Ser3029, reacts rapidly with the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF), and that alteration of this residue by site-directed mutagenesis caused a > 100-fold decrease in the rate of triketide lactone synthesis by chimaeric DEBS1–TE *in vivo* [12]. We therefore anticipated that PMSF would affect chain release in the *in vitro* system, and indeed addition of 1mM PMSF reduced production of the radiolabelled compound **1B** to 65% of the amount produced in the control incubation (data not shown). Cerulenin is an inhibitor of fatty acid synthases from almost all sources [15], and also inhibits production of compound **1B**. Compared to a control incubation, the presence of 0.1 mM cerulenin reduced the rate of incorporation 2-fold, and 1 mM cerulenin reduced the rate 10-fold (data not shown). Cerulenin probably exerts its effect through specific covalent modification of the active site of one or both of the ketoacyl-ACP synthase activities in DEBS1, as has been previously demonstrated for fatty acid synthase [16].

Relaxed specificity for the starter unit

The data in Figure 2 illustrate another feature of the cell-free enzymatic synthesis of triketide lactone. An additional major radiolabelled species is produced in parallel with compound **1B**. This product co-migrates on TLC and HPLC with an authentic synthetic sample of compound **1A**, the triketide lactone derived from use of an acetate rather than a propionate starter unit. The relative proportions of compounds **1B** and **1A** may be altered in the expected direction by the addition of either acetyl-CoA or greater amounts of propionyl-CoA to the standard reaction mixture (data not shown). The results are consistent with the idea that the cell extract contains both endogenous acetyl-CoA and propionyl-CoA, and that additional amounts of propionyl-CoA may be derived by *in situ* decarboxylation of methylmalonyl-CoA. An engineered strain of *S. coelicolor* containing the DEBS1-thioesterase has been shown previously to produce a mixture of compounds **1A** and **1B**, with compound **1A** as the major product [13]. For reasons that are not clear, DEBS1 alone, when expressed using the identical system in *S. coelicolor*, is reported by others to produce only compound **1B** [17].

Polyketide production by purified DEBS1–TE

The DEBS1–TE multienzyme was purified to 90–95 % homogeneity from *S. erythraea* JCB101 by methods that were previously successful for the native DEBS multienzymes [8,10] and for DEBS3 over-expressed in *Escherichia coli* [18]. The purified enzyme was used in another series of experiments to establish the extent to which alternative starter units could be used by the polyketide synthase. The incubations contained [^{14}C]-methylmalonyl-CoA and either acetyl-, propionyl-, *n*-butyryl-, *iso*-butyryl-, *n*-valeryl-, *iso*-valeryl-, *n*-hexanoyl- or crotonoyl-CoA, and the products were extracted and analyzed as before. As summarized in Figure 3, substantial amounts of new product were obtained from the incubations containing acetyl-, *n*-butyryl- and *iso*-butyryl-CoA, while all product mixtures also contained some propionate-derived material due to partial decarboxylation of the methylmalonyl-CoA. The radioactive products **1A**, **1B** and **1C** all co-migrated with authentic synthetic materials in three different HPLC and TLC systems.

Based on these results, it appears that the purified DEBS1–TE multienzyme not only retains its activity, but that it requires no extraneous cofactors other than NADPH. NADH did not function at all as a cofactor (data not shown). These data provide firm evidence for *in vitro* synthesis of polyketide chains on a purified modular PKS.

Stereospecificity of polyketide chain extension

We have recently shown that acylation of the methylmalonyl-CoA:acyl carrier protein acyltransferase (AT) domains from all six modules of DEBS is highly specific for the (2*S*)- stereoisomer of methylmalonyl-CoA [7].

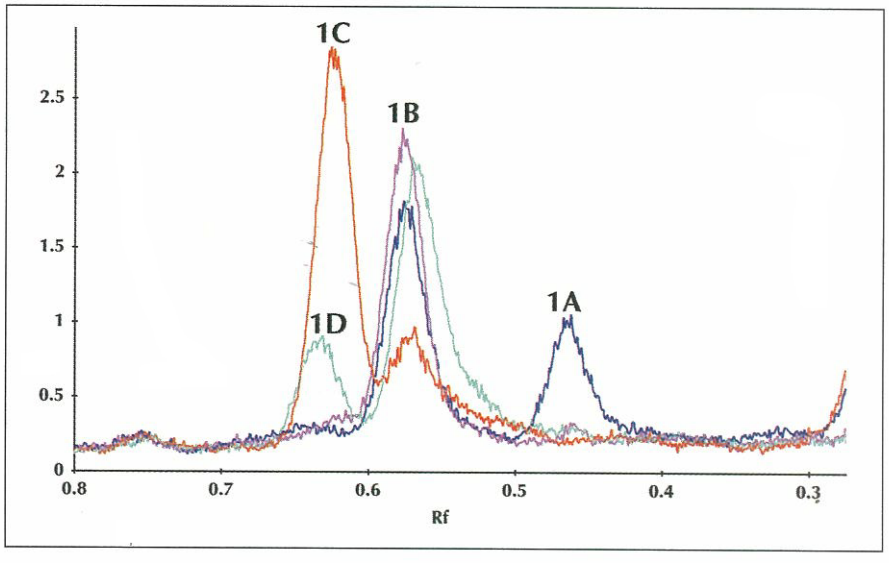

Fig. 3. A variety of starter units can be efficiently used by purified DEBS1–TE. The figure shows the production of different ^{14}C-labelled lactones **1(A–D)** by purified DEBS1–TE from the corresponding starter acyl-CoA. The lactones were separated on silica TLC plates and radioactivity was detected by two-dimensional phosphorimaging.

The acyl group is subsequently transferred to water in the absence of polyketide chain growth. The availability of purified DEBS1–TE multienzyme provided the means to check whether the same stereospecificity is exercised during normal chain extension.

(*R,S*)-[^{14}C]-methylmalonyl-CoA was pre-incubated with varying amounts of transcarboxylase (which specifically decarboxylates the (2*S*)-isomer) under conditions that ensure that the reaction is irreversible [7] (Fig. 4). The reaction with DEBS1–TE was then initiated and the triketide lactone products were detected as before. The amount of triketide lactone product fell sharply as the amount of added transcarboxylase in the pre-incubation was increased (Fig. 4a). Addition of purified methylmalonyl-CoA epimerase (which specifically interconverts the two isomers of methylmalonyl-CoA) along with transcarboxylase, however, caused a significant increase in the amount of product compared to that obtained in the absence of epimerase (Fig. 4a). The simplest interpretation of these results is that the levels of polyketide synthesis are partly restored when the (2*S*)-isomer removed by the action of the transcarboxylase is restored by generation from the (2*R*)-isomer. The complementary experiment was also carried out, in which methylmalonyl-CoA mutase [19] (which specifically uses (2*R*)-methylmalonyl-CoA as a substrate) was added in increasing amounts under conditions where the succinyl-CoA product is irreversibly hydrolyzed to succinate [7]. The loss of the (2*R*)-isomer had almost no effect on the amount of triketide lactone formed (Fig. 4b). When epimerase was added, causing (2*S*)-methylmalonyl-CoA to be depleted as well, the amount of lactone formed dropped to 35 % of the amount found in the absence of this enzyme. We conclude that (2*S*)-methylmalonyl-CoA specifically promotes polyketide synthesis whereas the 2*R* isomer does not, and that the stereospecificity we previously observed for the partial hydrolytic reactions on DEBS is also seen for chain synthesis. In other words, the PKS requires (2*S*)-methylmalonyl-CoA as an extender unit at all AT domains. This means, as previously proposed [7], that the stereochemical variation observed in complex macrolides must be the outcome of additional, as yet unidentified, enzymatic steps on the synthase.

Mass spectrometric analysis of triketide lactones 1B and 1C

High resolution mass spectrometric analysis was used to confirm the identity of the triketide lactones generated *in vitro*. Cell-free extracts of *S. erythraea* contain no *n*-butyryl-CoA, making this a convenient starter unit to monitor turnover of unlabelled substrates into triketide lactone **1C**. The products of a standard incubation in which *n*-butyryl-CoA replaced propionyl-CoA were extracted and purified by HPLC. Material eluting at the same position as authentic **1C** was analyzed by high-resolution electrospray mass spectrometry, in the absence (Fig. 5a) or presence (Fig. 5b) of added sodium ions. The addition of sodium ions produces a shift in the molecular ion peak and a reduction in the amount of dehydration occurring in the spectrometer. The masses of the principal peaks in the spectra were in close agreement with the values expected for the molecular ion of protonated compound **1C** (MH^+= 187.2), for a dehydrated species (MH^+-18 = 169.0), and for the

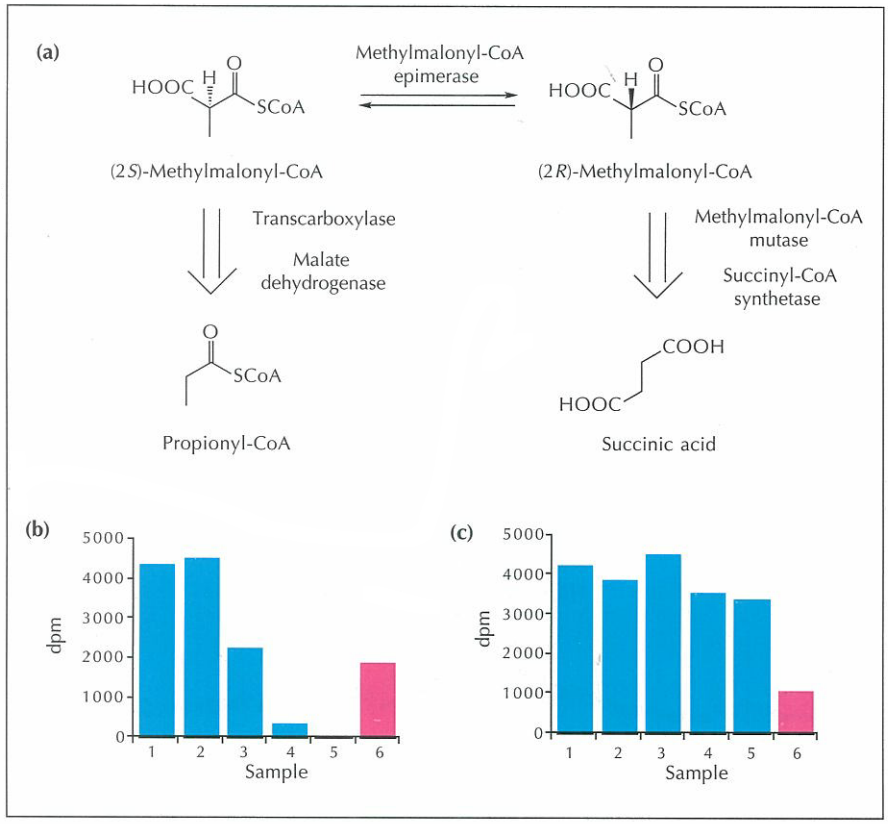

Fig. 4. Triketide lactone production by purified DEBS1–TE is stereospecific. **(a)** Reaction schematic. **(b)** When (2*S*)-methylmalonyl-CoA was selectively removed from the (*RS*) mixture by increasing amounts of transcarboxylase, the efficiency of [^{14}C]-triketide lactone production is progressively decreased: sample 1, no transcarboxylase; 2, 0.2 mU; 3, 2 mU; 4, 20 mU; 5, 200 mU; sample 6 as sample 5, but with 200 mU methylmalonyl-CoA epimerase. **(c)** Selective removal of (2*R*)-methylmalonyl-CoA by increasing amounts of methylmalonyl-CoA mutase had no effect: sample 1, no methylmalonyl-CoA mutase; 2, 0.2 mU; 3, 2 mU; 4, 20 mU; 5, 200 mU; sample 6 as sample 5, but with 200 mU methylmalonyl-CoA epimerase.

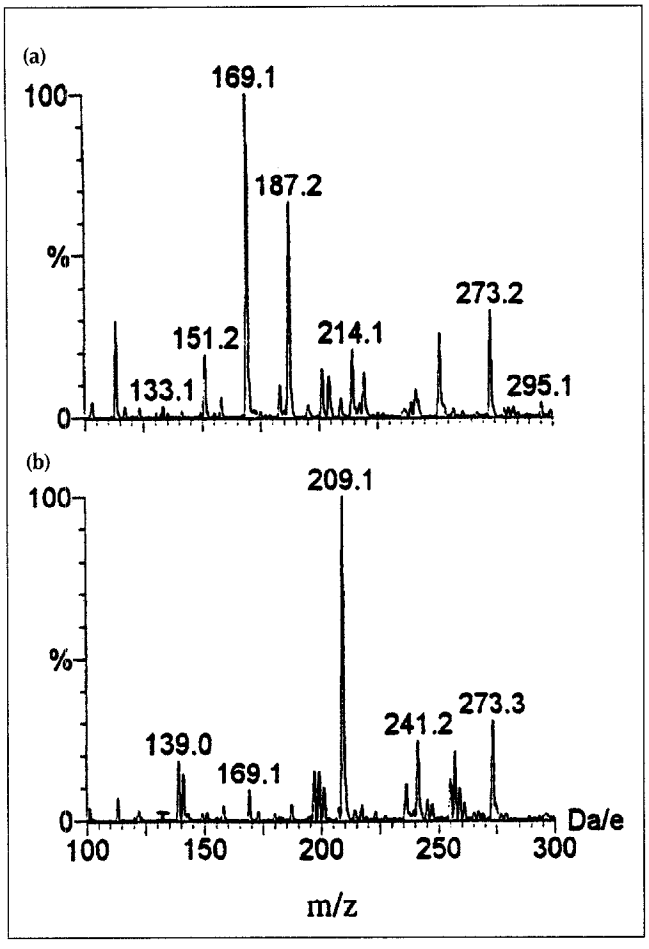

Fig. 5. Identification of lactone **1C** produced *in vitro* by electrospray mass spectrometry. **(a)** Spectrum in acid conditions showing the molecular ion MH^+ (187.2) and a dehydration product MH^+-18 (169.1). **(b)** Spectrum in the presence of Na^+ ions, showing the ion MNa^+ (209.1).

sodium adduct of compound **1C** (MNa^+= 209.1). The two molecular ion species were analyzed by high resolution mass spectrometry and gave excellent agreement with the required masses (found 187.13423, $C_{10}H_{19}O_3$ requires 187.13342; found 209.11533, $C_{10}H_{18}O_3Na$ requires 209.11539).

Confirmation of the identity of the triketide lactone **1B** synthesized *in vitro* was obtained by using specifically-deuterated propionyl-CoA as a substrate. To reduce competition by endogenous sources of starter units, the *S. erythraea* extract was depleted of small molecules by gel filtration. The products of the incubation were extracted with ethyl acetate, purified using reversed-phase HPLC, and the product with the elution volume of authentic **1B** was subjected to high-resolution electrospray mass spectrometry. The exact mass was determined to be 176.13605, in excellent agreement with the value expected for trideuterio-**1B** ($C_9{}^1H_{14}{}^2H_3O_3$ requires 176.13636). Another peak was observed in the mass spectrum at 158.12633, in similarly close agreement with the mass value expected for loss of water from trideuterio-**1B** ($C_9{}^1H_{12}{}^2H_3O_3$ requires 158.12652).

These data taken together firmly establish that *de novo* synthesis of compound **1C** and trideuterio-**1B** is occurring in the cell-free system, the first unequivocal demonstration of polyketide chain extension on a modular type I PKS *in vitro*.

Significance

The modular type I polyketide synthases of *Streptomyces* and related Gram-positive bacteria constitute a unique family of carbon-chain-forming multienzymes that are responsible for the biosynthesis of a large number of structurally-diverse natural products, including antibiotics and immunosuppressants. The most intensively-studied of these multienzymes, the 6-deoxy-erythronolide B synthase of *Saccharopolyspora erythraea*, is one of the simplest, with three different multienzyme subunits housing a total of about 30 active sites. Even for this relatively simple synthase, however, the complexity of the enzymology has frustrated previous attempts to demonstrate polyketide synthesis *in vitro*.

We have recently introduced a method of specifying the length of polyketide chain released from a modular polyketide synthase by using genetic manipulation to relocate the chain-terminating cyclase domain (TE) from DEBS3 to the carboxyl-terminus of DEBS1. Strains of *S. erythraea* harbouring the fused DEBS1–TE efficiently accumulate the anticipated triketide lactone.

We have now purified DEBS1–TE from such a strain and used it to synthesize triketide lactones *in vitro*. We have shown that the specificity for the starter unit is more relaxed than *in vivo*, and confirmed that the enzyme specifically uses the (2*S*)-isomer of methylmalonyl-CoA for chain extension. The demonstration that *in vitro* synthesis of polyketides is possible opens the way for detailed mechanistic studies of the modular polyketide synthases. Such systems, both *in vivo* and *in vitro*, are of great current interest for synthesis of novel polyketides of potential utility as lead compounds in drug discovery.

Materials and methods

Materials

Coenzyme A esters were purchased from Sigma. (*R,S*)-[3-^{14}C]-methylmalonyl-CoA was purchased from NEN. [^{14}C]-propionyl-CoA and [3-$^2H_3$]-propionyl-CoA were synthesized from [^{14}C]-propionic acid and [3-$^2H_3$]-propionic acid using *S*-acetyl-CoA synthetase, coenzyme A and ATP [7], purified by Mono-Q (Pharmacia) anion exchange chromatography followed by desalting on Sephadex G-10 . Purity was checked by TLC on cellulose in *n*-butanol:acetic acid:water (5:2:3 by vol.) followed by staining with sodium nitroprusside. Negative ion mass spectrometry using a VG BIO-Q mass spectrometer showed a molecular ion at 825, as expected for [3-$^2H_3$]-propionyl-CoA.

*Preparation of [^{14}C]-triketide lactone **1B***

A 250-ml flask containing 100 ml sucrose–succinate defined medium was inoculated with *S. erythraea* TED8 spores [12]. After 12 days, the cells were harvested and resuspended in 100 ml 3-(N-morpholino)-propanesulfonic acid:KOH buffer, pH 7.0. Sodium [1-^{14}C]-propionate (4 μCi) was added. After incubation for a further 24 h the cells were harvested and the supernatant extracted with two 100-ml volumes of ethyl acetate, dried over $MgSO_4$ and concentrated. When analyzed by TLC and HPLC, this material appeared identical to authentic [^{14}C]-labelled compound **1B** [13].

Growth of cells, preparation of the extract and purification of DEBS1–TE

Sucrose–succinate defined medium (10 l) was inoculated with a 72 h culture of *S. erythraea* JCB101 grown from spores in 400 ml M1-102 medium. *S. erythraea* JCB101 is a variant of *S. erythraea* TER43 [12] that produces slightly higher levels of triketide lactones. After 36 h of growth at 30 °C and 15 l min^{-1} aeration, cells were harvested by filtration. Cell paste (2.5 g) was homogenized in 10 ml 50 mM Tris-HCl buffer pH 7.5, 1 mM EDTA, 20 % v/v glycerol, 2 mM dithiothreitol, 1 mM phenanthroline, 1 mM benzamidine, 20 μg ml^{-1} trypsin inhibitor, 100 μg ml^{-1} DNase I and 50 μg ml^{-1} RNase A. Cells were broken at 1.1 GPa in a French pressure cell and the lysate clarified by ultracentrifugation at 100 000 g for 30 min at 4 °C.

Desalting of the extract, where required, was performed on Sephadex G-25 M. Purification of the DEBS1–TE was performed essentially as described for DEBS multienzymes from *S. erythraea* [8] and for over-expressed DEBS3 in *E. coli* [18]. Full details of the purification and characterization of DEBS1–TE will be published separately.

Assay conditions

Assays were carried out in the presence of 300 μM propionyl-CoA, 600 μM (*R,S*)-methylmalonyl-CoA, 600 μM NADPH, 14.6 μunits methylmalonyl-CoA epimerase per ml extract [14]. After incubation at 30 °C for 90 min, the reaction was stopped by snap-freezing in a dry ice/acetone bath. Reaction mixtures were acidified to pH 3.0 with 100 mM H_2SO_4 and extracted twice with an equal volume of ethyl acetate. Acetyl-CoA, *n*-butyryl-CoA, *iso*-butyryl-CoA, *n*-valeryl-CoA, *iso*-valeryl-CoA, *n*-hexanoyl-CoA and crotonoyl-CoA were used in some assays instead of propionyl-CoA .

For assays on a small scale, 100-μl aliquots of cell extract were added to the above components in a final volume of 120 μl. With purified enzyme, 125 μg of protein was used in the same final volume of 50 mM Tris–HCl buffer pH 7.5, 1 mM EDTA, 20 % v/v glycerol, 2 mM dithiothreitol. These small-scale assays included 27 nCi [3-^{14}C]-(*R,S*)-methylmalonyl-CoA. Products were separated by TLC on silica plates, eluted twice with diethyl ether, and two-dimensional scanning was performed with a Molecular Dynamics Phosphorimager™ 425. Normal-phase HPLC was carried out on a Hewlett Packard HP1090 instrument, using a Spherisorb SW5 column (PhaseSep, 5 μm, 25 cm x 4.6 mm) developed isocratically with diethyl ether/cyclohexane (1:1 v/v) at a flow rate of 1 ml min^{-1}. Reversed-phase HPLC was carried out on a Spherisorb S5CN column (PhaseSep, 5 μm, 25 cm x 4.6 mm) developed with a linear gradient from methanol/water (1:9 v/v) to methanol/water (1:1 v/v) at a flow rate of 1 ml min^{-1}. Where appropriate, 0.5 ml fractions were collected and the radioactivity counted using Optiphase 'Hisafe'2 scintillant in a Canberra Packard 2000CA Tricarb Liquid Scintillation Counter.

Large-scale assays with $^2H_3$-propionyl-CoA or *n*-butyryl-CoA, for which no radiolabel was required, were carried out as described above but in a final volume of 22 ml. Products were separated by HPLC as before and analyzed by mass spectrometry on a VG-BIOQ mass spectrometer.

The stereospecificity of polyketide chain extension

To remove the (2*S*)-isomer of methylmalonyl-CoA, 2.4 mM (*R,S*)-methylmalonyl-CoA (containing 27 nCi (*R,S*)-[3-^{14}C]-methylmalonyl-CoA) was incubated for 20 min at 30°C in the presence of 100 units (U) malate dehydrogenase, 11 mM NADH, 11 mM sodium pyruvate and either 200 mU, 20 mU, 2 mU, 0.2 mU or 0 mU of transcarboxylase, respectively, in a final volume of 100 μl 50 mM Tris-HCl buffer pH 7.5. After 20 min, propionyl-CoA (final concentration 300 mM), NADPH (final concentration 600 μM) and 125 μg DEBS1–TE (90 % pure) were added and the 200-μl mixture was incubated for 60 min. To a parallel incubation with 200 mU transcarboxylase, 200 mU methylmalonyl-CoA epimerase was added simultaneously with the DEBS1–TE.

To remove the (2*R*)-isomer, 2.4 mM (*R,S*)-methylmalonyl-CoA (containing 27 nCi (*R,S*)-[3-^{14}C]-methylmalonyl-CoA) was incubated for 20 min at 30°C in the presence of 10 mM $MgCl_2$, 0.1 mM EDTA, 4 mM arsenate, 0.23 U succinyl-CoA synthetase (the kind gift of Professor J. Nishimura, University of Texas), 5 μM coenzyme B_{12} and 200 mU, 20 mU, 2 mU, 0.2 mU and 0 mU respectively of methylmalonyl-CoA mutase in a final volume of 100 μl 100 mM Tris-HCl, pH 8.0. After 20 min, assays were carried out as above. To another incubation with 200 mU mutase, 200 mU methylmalonyl-CoA epimerase was added and after a further 20 min incubation at 30°C the assay was started with the addition of propionyl-CoA, NADPH and DEBS1–TE .

Acknowledgements: This work was supported by grants from the Biomolecular Sciences Committee of the Engineering and Physical Sciences Research Council (EPSRC) and the Biotechnology and Biology Research Council (BBSRC), and from the Chemicals and Pharmaceuticals Directorate of the BBSRC to P.F.L. and J.S.; also by a Postgraduate Studentship from BBSRC to K.E.H.W. and a Postgraduate Studentship from Ciba-Geigy PLC to A.L.C. P.F.L. and J.S. are members of the Cambridge Centre for Molecular Recognition.

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Received: **18 July 1995**. Accepted: **2 August 1995**.