

# Novel octaketide macrolides related to 6-deoxyerythronolide B provide evidence for iterative operation of the erythromycin polyketide synthase

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**Background:** The macrolide antibiotic erythromycin A, like other complex aliphatic polyketides, is synthesised by a bacterial modular polyketide synthase (PKS). Such PKSs, in contrast to other fatty acid and polyketide synthases which work iteratively, contain a separate set or module of enzyme activities for each successive cycle of polyketide chain extension, and the number and type of modules together determine the structure of the polyketide product. Thus, the six extension modules of the erythromycin PKS (DEBS) together catalyse the production of the specific heptaketide 6-deoxyerythronolide B.

**Results:** A mutant strain of the erythromycin producer *Saccharopolyspora erythraea*, which accumulates the aglycone intermediate erythronolide B, was found unexpectedly to produce two novel octaketides, both 16-membered macrolides. These compounds were detectable in fermentation broths of wild-type *S. erythraea*, but not in a strain from which the DEBS genes had been specifically deleted. From their structures, both of these octaketides appear to be aberrant products of DEBS in which module 4 has 'stuttered', that is, has catalysed two successive cycles of chain extension.

**Conclusions:** The isolation of novel DEBS-derived octaketides provides the first evidence that an extension module in a modular PKS has the potential to catalyse iterative rounds of chain elongation like other type I FAS and PKS systems. The factors governing the extent of such 'stuttering' remain to be determined.

## Introduction

Fatty acid synthases (FASs) provide the paradigm of a multienzyme system that sequentially adds units to a growing chain by the repeated operation of a single set of enzyme active sites, before release of the final product. Although the protein architecture of FAS multienzymes varies from the multifunctional polypeptides (type I systems) in fungi and animals [1], to the dissociable monofunctional enzymes (type II systems) of most bacteria [2], the mechanism of iterative chain extension is essentially the same. Similarly, the type II polyketide synthases (PKSs) that catalyse the production of aromatic polyketides in bacteria do so by simple, repetitive use of a core set of FAS-related enzyme activities [3]. Fungal type I PKSs, as first shown for 6-methylsalicylic acid synthase (MSAS) of *Penicillium patulum* [4], also contain a single set of enzymes, even though (by mechanisms that are not yet understood) the degree of reduction accomplished during each successive cycle may be different.

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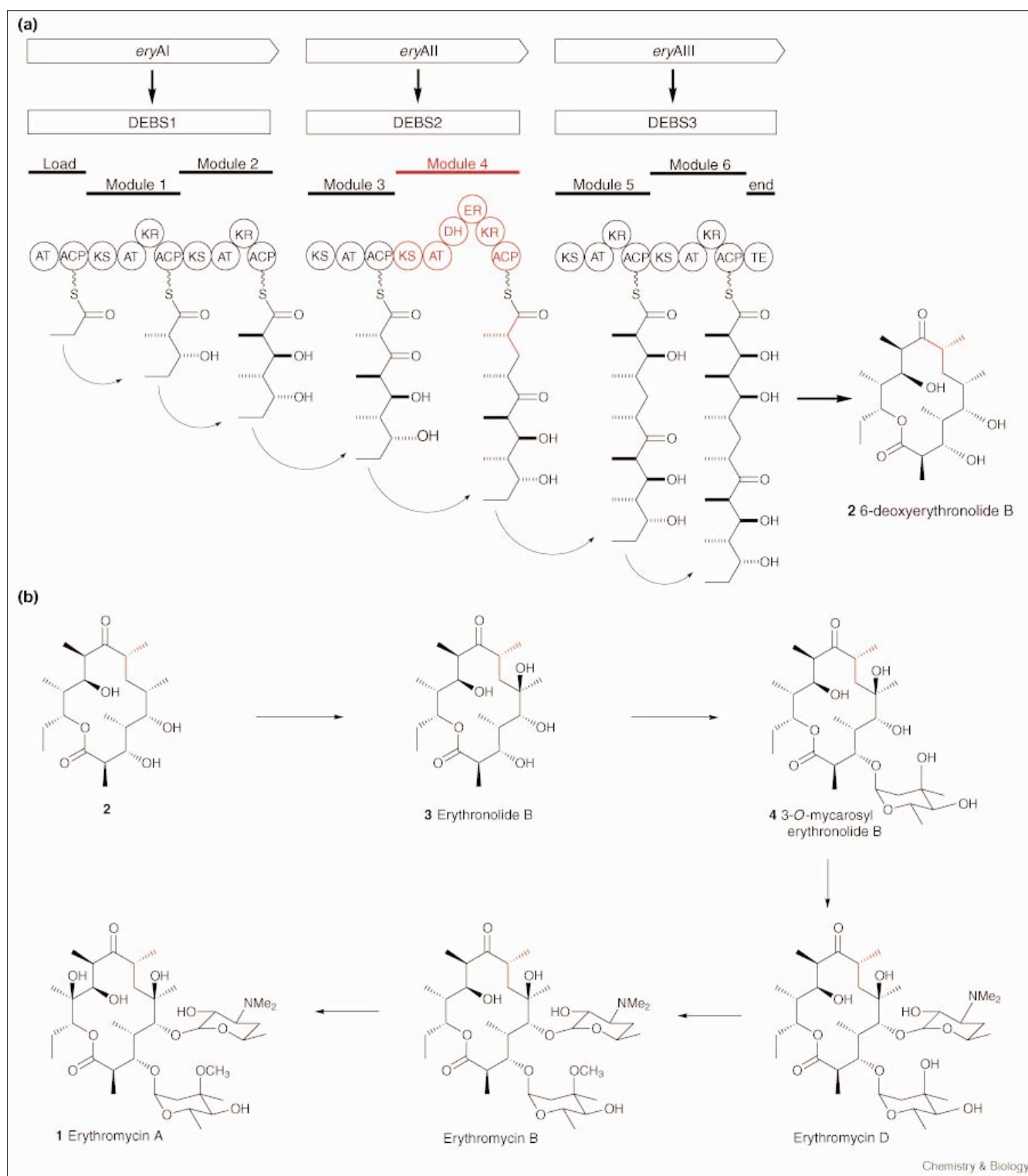
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In contrast, complex macrocyclic polyketides such as the antibacterial erythromycin A **1** and the immunosuppressant rapamycin are synthesised, in *Streptomyces* and related bacteria, on modular type I PKS assemblies, in which a different set or module of enzymes is used to catalyse each successive cycle of chain extension [5,6]. Thus, the polyketide synthase responsible for production of the aglycone 6-deoxyerythronolide B (6-dEB) **2** catalyses the decarboxylative condensation of six (2S)-methylmalonyl-CoA extender units [7] with a propionate starter unit derived from propionyl-CoA. The 6-dEB synthase (DEBS) consists of three multifunctional proteins (DEBS1, DEBS2 and DEBS3) [8], each of which houses two extension modules (Figure 1a), although in other systems the number of modules in a single protein may vary from one [9] to six [10,11]. This linear, processive mechanism appears to be general for the biosynthesis of aliphatic polyketides by type I PKSs in bacterial systems [5,6,12–19], as well as for nonribosomal peptide biosynthesis [20]. For DEBS, the loading

Figure 1



**(a)** The erythromycin-producing PKS; primary organisation of the genes and their corresponding protein domains. There are six chain extension modules each beginning with a  $\beta$ -ketoacyl synthase domain that are responsible for adding the six successive extension units. The completed chain is released as a heptaketide macrolactone by the

thioesterase domain. AT, acyltransferase; ACP, acyl carrier protein; KS,  $\beta$ -ketoacyl synthase; KR,  $\beta$ -ketoacyl reductase; DH, dehydratase; ER, enolyreductase; TE, thioesterase. **(b)** The post-PKS modifications of 6-dEB (**2**) which give rise to erythromycin A (**1**) in the *S. erythraea* NRRL2338 wild-type parental strain.

module (the acyl transferase–acyl carrier protein (AT–ACP) bidomain at the amino terminus of DEBS 1) [21] and the six extension modules together produce an enzyme-bound heptaketide chain, which is subsequently cyclised to the first enzyme free intermediate 6-dEB **2** through the action of a thioesterase domain (TE) at the carboxyl terminus of DEBS 3. Several post-PKS steps, including hydroxylation, glycosylation and methylation, finally yield the active antibiotic **1** (Figure 1b) [22].

The chain length of the polyketide product of DEBS has been altered to generate diketide [23], triketide [24], tetraketide [25] and hexaketide [26] products. In these experiments, the terminating thioesterase was re-located to the end of a PKS gene assembly containing the requisite number of extension modules, whereas in other work combinations of modules have been assembled to yield specific polyketide products [27,28]. The products formed by both natural and engineered PKSs have thereby been shown to be dictated by the number and type of modules in the PKS responsible. In a few cases, a ‘shorter’ polyketide product has been observed [13,29] and attributed to premature release of the growing chain during the extension process. However, bacterial modular type I PKSs have never been reported to produce longer products, through the iterative use of one or more extension module(s).

As part of our work aimed at the production of novel polyketide metabolites through engineered (combinatorial) biosynthesis [22,30–33], a detailed chromatographic characterisation has been performed of the fermentation products produced both by an erythromycin-producing strain of *Saccharopolyspora erythraea* and also by mutants of *S. erythraea* specifically deficient in either 6-dEB production or in glycosylation of the macrolide aglycone. Such *S. erythraea* strains, in conjunction with the recently-described *Escherichia coli*/*Streptomyces* shuttle vector pCJR24 [34], provide useful hosts for heterologous expression of hybrid PKSs. Particular attention was paid to identifying minor compounds which are often formed as shunt products of biosynthetic pathways. In this way two new octaketide macrolides have been identified and characterised as products of a mutant of *S. erythraea* NRRL2338, and have then been detected in the wild-type strain. The structures of these new products indicate that module 4 of the DEBS multienzyme is capable of catalysing two (iterative) rounds of chain elongation, a phenomenon we refer to as ‘stuttering’.

## Results

### Two novel 16-membered macrolides from *S. erythraea*

The mutant *S. erythraea* strain number 5 (whose construction will be described elsewhere) specifically lacks the ability to convert erythronolide B into later metabolites in the erythromycin biosynthetic pathway. The mutant *S. erythraea* strain JC2 lacks the *eryAI–AIII* genes that

govern production of DEBS, and it therefore produces no erythromycins, but retains, functionally intact, all the genes responsible for post-PKS modification of **2** to **1** [34]. The fermentation of these strains, and of the wild-type erythromycin-producing strain, was performed in two stages, a seed stage (SV2 medium), followed by a production stage (SM3 medium). Methanol extracts of whole broth and ethyl acetate extracts of culture supernatants were subjected to liquid chromatography–mass spectroscopy (LC–MS) analysis using both electrospray (ES) and atmospheric-pressure chemical-ionisation (AP–CI) techniques.

The analysis of extracts from the fermentation of *S. erythraea* number 5 showed the presence of significant amounts of several unknown species, albeit at low levels when compared with 6-dEB **2** or EB **3**. Further analysis of two such components, eluting close together and with parent masses of 414 and 428 revealed distinctive LC–MS–MS spectra [35] indicative of polyketide macrolide products derived from acetate and propionate starter units, respectively. The ability of the DEBS1 loading bidomain to accept both propionate and acetate starter units has been well documented [36].

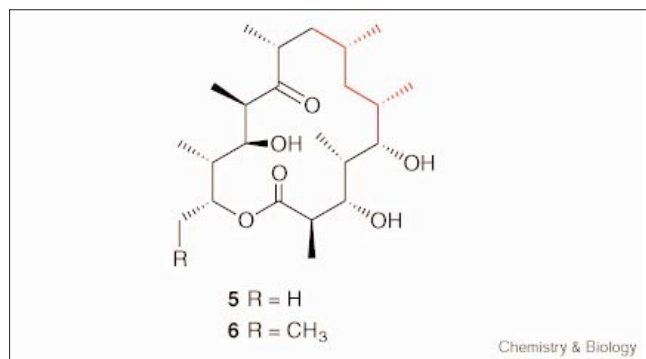
The two unknown compounds were extracted from a 31 fermentation of *S. erythraea* number 5, and shown, using LC–MS, to represent about 1% of the amounts of **2** and **3** present. The two compounds were isolated after repeated rounds of preparative-scale reverse-phase high-performance liquid chromatography (HPLC), but in low yields (0.5–1 mg each). The combined titer of **2** and **3** from a typical fermentation was approximately 50 mg/l.

The molecular weights of the two new compounds **5** and **6** (Figure 2) were obtained using LC–ES–MS, and the molecular formulae determined from high-resolution ES–TOF–MS data. Their structural analysis was completed using one-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectroscopic methods, with the long range  $^1\text{H}$ – $^{13}\text{C}$  correlations from the regularly spaced methyl groups being most helpful. To our surprise the data readily identified **5** and **6** as octaketides, both of them being 16-membered macrolides. These structures are clearly related to that of 6-dEB **2**, except that the normal fourth extension cycle seems to have been repeated.

### Proposed mechanism for formation of octaketide 16-membered macrolides by ‘stuttering’ on the DEBS multienzyme

It has been shown previously that a suitably functionalised synthetic triketide can be recognised as a diketide analogue by DEBS and elongated to an octaketide [37]. However, the *de novo* biosynthesis of an octaketide by a native hexamodular PKS, as observed here, is unprecedented. The structures of the observed octaketides

Figure 2



The compounds **5** and **6**, which we believe occur when module 4 of DEBS2 catalyses an additional iterative round of chain elongation. The absolute stereochemistries are inferred from that of 6-dEB **2**.

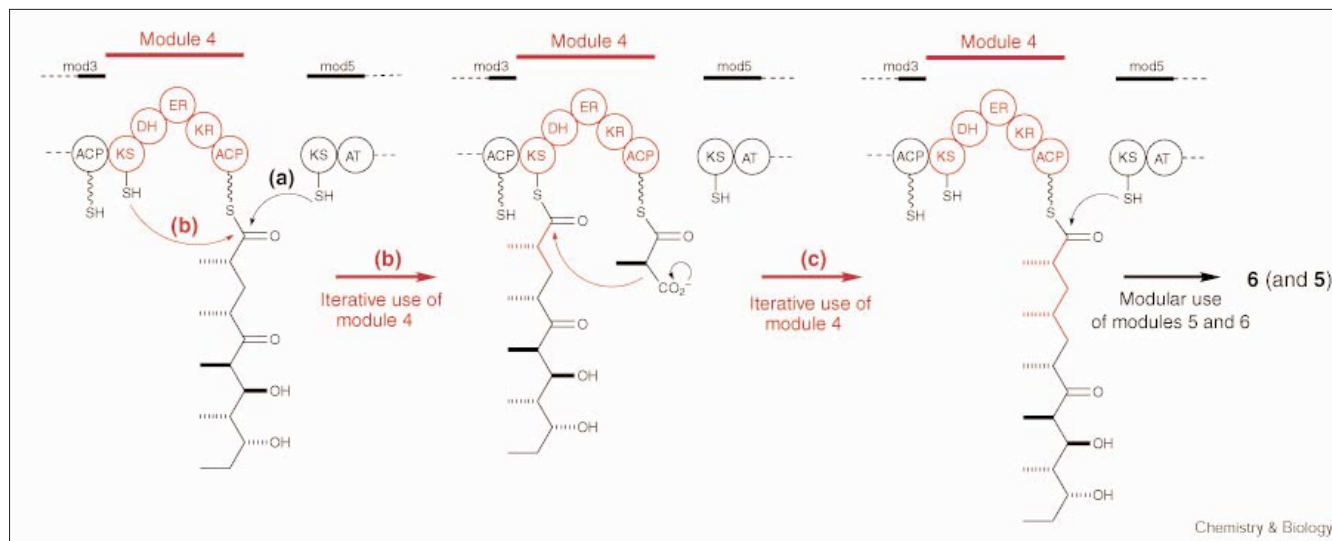
immediately suggested that the octaketides might represent aberrant products of the DEBS PKS, where the PKS had 'stuttered' at the end of DEBS module 4, that is, had catalysed an additional (iterative) round of chain elongation before transfer of the chain to module 5 in DEBS3 (Figure 3). Formation of **5** and **6** must be catalysed by DEBS module 4, because module 4 is the only module in DEBS that carries the full complement of reductive domains required for the formation of a methylene group. This homologation reaction would require the normal pentaketide product of module 4, attached to the ACP, to be transferred onto the active-site cysteine residue within the  $\beta$ -keto acyl synthase (KS) domain of the same

module. A second module-4-catalysed chain extension event would then occur, producing a hexaketide chain attached to the ACP of module 4, which would then be transferred to the KS of module 5 (Figure 3).

On the basis of a proposed helical structure of DEBS [38], we can attempt to describe the movement of the stuttering chain within module 4. This helical model for DEBS is the only one in accordance with all the published structural and mechanistic data available [38–41]. The helical model predicts a structure for DEBS2 (Figure 4) in which a DEBS2 homodimer has modules associated head-to-head and tail-to-tail that twist together to form a rope-like quaternary structure. The core KS, AT and ACP domains are associated along the axis of the structure with the reductive domains present as loops to the sides. One clear indication from this model is that the KS4 domain of one chain can interact only with ACP4 of the opposite chain. This is in agreement with data from chemical-cross-linking and proteolysis studies [38,39] and from mutant complementation [40,41]. Thus, in order to perform the second chain extension event, and subsequent reduction to a methylene group, the 'stuttering' chain must be passed back to the same KS4 domain that catalysed the initial chain extension event. As such, chain elongation during 'stuttering' is truly iterative.

It cannot be ruled out that other minor metabolites, which correspond to iterative use of one of the other five DEBS modules, are produced even though they were not detected in our experiments. The isolation of the particular compounds **5** and **6** may, for example, reflect higher yields

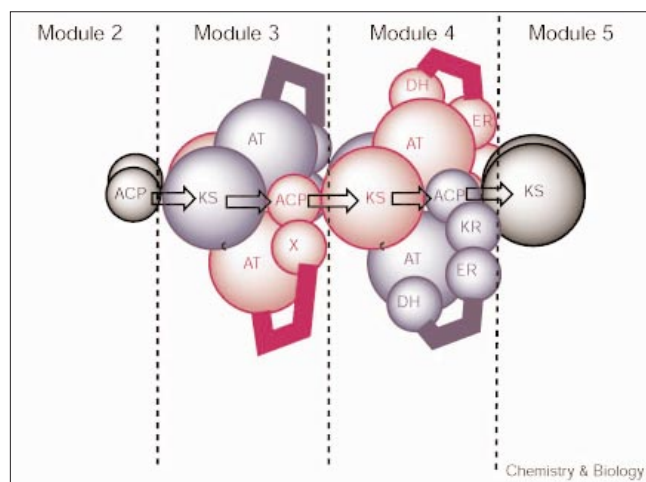
Figure 3



The normal pentaketide product of module 4 can either process the growing chain to the KS domain of module 5 (**a**) in the normal manner to produce after two further rounds 6-dEB **2**, or it can reacylate the KS domain of module 4 (**b**), which then undergoes a second chain

extension event on this module (**c**) before processing the new hexaketide to module 5 thereby giving rise to the iterative product **6** through 'stuttering' of DEBS.

Figure 4



Three-dimensional representation of DEBS2 based on the proposed helical model [38]. The domains are shown as spheres, and the two chains of the homodimer are coloured differently. The arrows show the movement a processing polyketide chain would take along DEBS2, which clearly shows that the KS and AT domains of opposite chains of the synthase must co-operate with one another, and not with those of the same chain.

arising from the position of module 4 at the end of a PKS multienzyme, where stuttering may compete more effectively with intermodular transfer between different DEBS multienzymes, than elsewhere in DEBS with intraprotein transfers between modules.

#### Octaketides are not produced by mutant *S. erythraea* lacking the DEBS genes

An alternative possibility for the origin of **5** and **6** could be the presence of a second PKS gene cluster in *S. erythraea* that is closely related to DEBS but has an additional (seventh) extension module. Such a PKS has not been identified following the careful analysis of total genomic DNA from this strain using PKS-specific hybridisation probes, but as a direct check we examined the fermentation supernatant of the *S. erythraea* mutant strain JC2 [34]. In this mutant the genes for almost the entire DEBS PKS have been specifically removed. After the LC–MS analysis of extracts from 24 individual *S. erythraea* JC2 single colonies, each fermented in two different media (complex and defined), no compounds corresponding to **5** and **6** could be observed. The data were verified by spiking each of the extracts with solutions of authentic **5** and **6**. When these experiments were repeated in the presence of added 6-dEB **2**, both **5** and **6** remained undetectable, whereas **2** was readily converted to **1**, demonstrating that a later intermediate in erythromycin biosynthesis is not an essential trigger for formation of **5** and **6**.

The failure to detect **5** and **6** in these experiments was shown to be significant using *S. erythraea* mutant strain

number 5, in which the DEBS genes are intact, but in which aglycone processing is deficient. Of 24 parallel fermentations of single colony isolates of the number 5 mutant, 14 of the clones produced **5** and **6** at levels similar to that which had led to their isolation, whereas several others produced them at reduced levels. These data taken together clearly implicate the DEBS proteins in the biosynthesis of **5** and **6**.

The analysis of 24 single colony fermentations of wild-type *S. erythraea* showed even lower levels of the two new compounds as judged using LC–MS. However, in over half of these we were able to identify **5** and **6** using LC–MS techniques, and confirmed that they co-eluted with authentic materials. The lower levels observed in fermentations of the wild-type strain may be a consequence of the presence of the full complement of aglycone-modifying enzymes. Some preliminary evidence (data not shown) was obtained, using LC–MS, for a species in which **6** may have been modified by hydroxylation and addition of a mycarosyl moiety (cf. compound **4**, Figure 1b).

#### Determination of chain length on modular PKS multienzymes

The results reported here demonstrate an apparent breakdown of ‘co-linearity’ between the number and type of modules in a type I modular PKS, and the chain length of the polyketide product. Previously reported experiments, in which hybrid polyketide products were obtained from engineered modular PKSs, have already shown that these systems can sometimes tolerate significant changes in substrate structure. Repeated chain extension upon a particular module, analogous to that observed for FASs and fungal PKSs [42,43], can therefore be viewed simply as another consequence of such imperfect molecular recognition. Indeed, the observation of stuttering emphasises that there is no fundamental mechanistic difference between modular type I PKS and iterative PKSs and FASs. Modular PKSs have presumably evolved to minimise stuttering, by ensuring that the KS domain of each extension module, vacated after the condensation step, is rapidly occupied by an incoming acyl chain so that the newly extended acyl chain (which after condensation is attached to the ACP) is prevented from migrating back to the KS domain of the same module. This view of catalysis on modular PKSs would imply the simultaneous extension of multiple chains on the PKS, which is in accord with recent experiments where blocking the transfer of the completed polyketide chain from the rifamycin PKS apparently leads to shedding of partially assembled polyketides of different length from every extension module [44].

#### Significance

Modular polyketide synthases (PKSs) normally exert a rigorous control over chain length, because each extension module in the multienzyme assembly line operates only once before handing on the extended polyketide

chain, and a thioesterase or other transferase precisely controls release of the full-length product. Similarly, truncated products are readily rationalised as the products of premature termination and release. However, we have shown here, for the hexamodular 6-deoxyerythronolide B (6-dEB) synthase (DEBS) from the erythromycin biosynthetic pathway, that low levels of octaketide products are also formed. The structures of these 16-membered lactones, when compared with those of the normal 14-membered lactones produced by this PKS, indicate that module 4 of DEBS has aberrantly operated twice in succession, a phenomenon we refer to as 'stuttering'. This unexpected observation underlines the close mechanistic connection between the modular PKSs and the wholly iterative fatty acid synthases and fungal PKSs, and provides additional insight into the mechanisms by which the modular PKS ensures fidelity of chain extension.

## Materials and methods

### Chemical analysis

NMR spectra were obtained using standard pulse sequences on a Bruker AMX 500 spectrometer fitted with a 3 mm Nalorac broad band probe. LC-MS analysis was performed on a Finnegan MAT LCQ-MS coupled to a Hewlett Packard 1050 HPLC integrated system. Analyses were run using either an ES or AP-Cl interface and using positive-negative ionisation mode switching. Chromatography was over a 5 micron Hypersil C<sub>18</sub> BDS column (150 mm × 4.6 mm i.d.) eluting at 1 ml/min with a gradient of 0–100% B over 30 min (A = 10 mM ammonium acetate + 0.1% formic acid; B = 90% acetonitrile:10%–10 mM ammonium acetate). Accurate mass data were obtained using a Micromass QTOF fitted with an ES source operated in the positive ion mode. The instrument was calibrated using a polyethylene glycol mixture (200/400/600/1000) with corrections for drift made with a lock mass of erythromycin A.

### Fermentation and extraction

*S. erythraea* NRRL2338 and the mutant *S. erythraea* strains JC2 and No.5 were maintained on plates of R2T20 agar at 30°C. Spores were harvested after ~2 weeks and stored in 10% glycerol at –80°C (spores from one plate were resuspended in 2 ml). Frozen spore suspension (100 µl) was used to inoculate 50 ml of SV2 seed medium in a 250 ml conical flask. SV2 medium contained per litre of deionised water: glucose (15 g), glycerol (15 g), soy peptone (15 g), NaCl (3 g) and CaCO<sub>3</sub> (1 g); adjusted to pH 7.0 before autoclaving. The seed culture was incubated at 250 rpm and 28°C for 3 days. This was then used to inoculate SM3 production medium (2% v/v) which was incubated at 28°C and 250 rpm. SM3 medium contained per litre of deionised water: glucose (5 g), maltodextrin (glucidex; 50 g), soya flour (arkasoy; 25 g), beet molasses (3 g), K<sub>2</sub>HPO<sub>4</sub> (0.25 g), CaCO<sub>3</sub> (2.5 g); adjusted to pH 7.0 before autoclaving. Larger-scale fermentations were carried out in 2 l Florence flasks containing 300 ml of SM3 medium. After 7 days the fermentation broths were harvested by centrifugation and the supernatant extracted with an equal volume of ethyl acetate containing ethanol (0.5% v/v). This was repeated twice, and solvents were removed from the combined extracts *in vacuo* to yield a viscous red oil. Analytical scale fermentations were carried out in the same way in miniature culture vessels containing 7 ml of SV2, SM3 or sucrose-succinate defined medium [24]. The oily residues from analytical fermentations were redissolved in methanol (100 µl), an equal volume of water was added and the mixture was then centrifuged to remove particulate matter before the supernatant (10 µl) was analysed using LC-MS.

### Isolation and purification of metabolites

The extract from 3 l of *S. erythraea* NRRL2338/no.5 fermentation broth (960 mg) was resuspended in a mixture of 1:1 methanol/water (6 ml) and filtered. The resulting solution was subjected to preparative HPLC-MS (Gilson 305/306 pumps, 811C mixer and 806 manometric module) (4 × 1.5 ml injections) using a 5 micron Hypersil Elite C<sub>18</sub> column (150 mm × 21.2 mm i.d.) eluted at 15 ml/min with a gradient of 0–100% solvent B over 75 mins, then held at 100% solvent B for 15 mins. Solvent A: milli-Q water plus 10 ml/litre 1 M ammonium acetate; Solvent B: 80% acetonitrile/water plus 20 ml/litre 1 M ammonium acetate. Fractions were collected every minute and the eluate was monitored at 220 nm, then split (1:400 ratio) for ES-MS analysis (Micromass LCZ platform.) The combined fractions containing **5** and **6** respectively were concentrated on Bond Elute 500 mg C<sub>18</sub> cartridges. After washing with water, **5** and **6** were eluted with methanol, which was then removed *in vacuo* and the residues (ca. 10 mg) were dissolved in acetonitrile (0.5 ml). These fractions were further purified (4 × 125 µl injections) by HPLC on a 5 micron Hypersil BDS C<sub>18</sub> column (150 mm × 4.6 mm i.d.) eluted with 70% acetonitrile/water containing 15 ml/litre 1 M ammonium acetate and monitored at 220 nm. The fractions were analysed by flow-injected ES-MS, and those fractions containing **5** or **6** respectively were combined and concentrated using Bond Elute 500 mg C<sub>18</sub> cartridges, which were washed with water. After elution with methanol, which was then removed *in vacuo*, ~0.5 mg each of **5** and **6** was obtained.

### GW479439x (5)

<sup>1</sup>H NMR (500 MHz, *d*<sub>6</sub>-DMSO) δ = 5.34 (dq, 1H, *J* = 6.5 & 1.0, 15-H), 4.80 (d, 1H, *J* = 6.0, 3-OH), 4.57 (d, 1H, *J* = 7.5, 13-OH), 4.05 (d, 1H, *J* = 5.0, 5-OH), 3.88 (ddd, 1H, *J* = 10.0, 7.5 & 2.5, 13-H), 3.54 (ddd, 1H, *J* = 8.5, 6.0 & 2.0, 3-H), 3.46 (ddd, 1H, *J* = 6.5, 5.0 & 2.5, 5-H), 2.90 (m, 10-H), 2.76 (dq, 1H, *J* = 7.0 & 2.5, 12-H), 2.51 (1H, 2-H), 1.60 (1H, 6-H), 1.57 (1H, 4-H), 1.53 (1H, 14-H), 1.47 (1H, 7-H), 1.40 (1H, 9-H), 1.19 (d, 3H, *J* = 6.5, 16-H), 1.12 (d, 3H, *J* = 7.0, 18-H), 1.01 (1H, 9-H), 0.96 (d, 3H, *J* = 6.5, 20-H), 0.95 (d, 3H, *J* = 7.0, 19-H), 0.95 (d, 3H, *J* = 7.0, 22-H), 0.91 (1H, 7-H), 0.88 (d, 3H, *J* = 7.0, 21-H), 0.88 (d, 3H, *J* = 7.0, 24-H), 0.87 (d, 3H, *J* = 7.0, 23-H).

<sup>13</sup>C NMR (100 MHz, *d*<sub>6</sub>-DMSO) 214.8 (C-11), 175.1 (C-1), 76.3 (C-5), 74.7 (C-3), 70.8 (C-13), 69.0 (C-15), 45.2 (C-12), 43.5 (C-2), 43.1 (C-9), 41.9 (C-14), 39.8 (C-4), 38.9 (C-10), 37.4 (C-7), 36.5 (C-6), 28.9 (C-8), 22.9 (C-21), 18.7 (C-20), 18.7 (C-16), 15.8 (C-22), 14.7 (C-17), 9.1 (C-24), 8.4 (C-19), 6.8 (C-23).

MS (ES-TOF) *m/z* C<sub>23</sub>H<sub>43</sub>O<sub>6</sub> calc'd 415.3060, found 415.3072 [MH]<sup>+</sup>. C<sub>23</sub>H<sub>42</sub>O<sub>6</sub>Na calc'd 437.2879, found 437.2896 [MNa]<sup>+</sup>.

### GW479438x (6)

<sup>1</sup>H NMR (500 MHz, *d*<sub>6</sub>-DMSO) δ = 5.17 (dd, 1H, *J* = 4.5 & 9.5, 15-H), 4.82 (brs, 1H, 3-OH), 4.50 (d, 1H, *J* = 7.0, 13-OH), 4.03 (brd, 1H, *J* = 4.5, 5-OH), 3.86 (ddd, 1H, *J* = 2.5, 7.0 & 10.0, 13-H), 3.52 (brd, 1H, *J* = 9.5, 3-H), 3.48 (brs, 1H, 5-H), 2.86 (m, 1H, 10-H), 2.74 (dq, 1H, *J* = 2.5 & 6.5, H-12), 2.56 (dq, 1H, *J* = 9.5 & 6.5, 2-H), 1.68 (1H, 16-H), 1.59 (1H, 14-H), 1.55 (1H, 6-H), 1.54 (1H, 4-H), 1.47 (1H, 8-H), 1.46 (1H, 16-H), 1.44 (1H, H-7), 1.38 (1H, H-9), 1.15 (d, 3H, *J* = 6.5, 18-H), 1.00 (1H, H-9), 0.97 (d, 3H, *J* = 7.0, H-20), 0.95 (d, 3H, *J* = 6.5, H-22), 0.94 (d, 3H, 7.0, H-19), 0.91 (1H, H-7), 0.88 (d, 3H, *J* = 6.5, H-21), 0.86 (d, 3H, *J* = 6.5, H-23), 0.84 (d, 3H, *J* = 7.0, H-24), 0.84 (t, 3H, *J* = 7.5, H-17).

<sup>13</sup>C NMR (100 MHz, *d*<sub>6</sub>-DMSO) δ = 213.5 (C-11), 175.5 (C-1), 76.2 (C-5), 74.8 (C-3), 74.5 (C-15), 71.0 (C-13), 45.3 (C-12), 43.5 (C-2), 43.3 (C-9), 40.6 (C-14), 39.8 (C-4), 38.8 (C-10), 37.2 (C-7), 36.9 (C-6), 29.1 (C-8), 25.9 (C-16), 22.9 (C-21), 18.7 (C-20), 15.9 (C-22), 15.5 (C-18), 9.8 (C-17), 9.5 (C-24), 8.4 (C-19), 6.7 (C-23).

MS (ES-TOF) *m/z* C<sub>24</sub>H<sub>44</sub>O<sub>6</sub>Na calc'd 451.3036, found 451.3020 [MNa]<sup>+</sup>. C<sub>24</sub>H<sub>48</sub>NO<sub>6</sub> calc'd 446.3482, found 446.3469 [MNH<sub>4</sub>]<sup>+</sup>.

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