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Research Paper

Engineering a polyketide with a longer chain by insertion of an extra module into the erythromycin-producing polyketide synthase

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Abstract

Background: Modular polyketide synthases catalyse the biosynthesis of medically useful natural products by stepwise chain assembly, with each module of enzyme activities catalysing a separate cycle of polyketide chain extension. Domain swapping between polyketide synthases leads to hybrid multienzymes that yield novel polyketides in a more or less predictable way. No experiments have so far been reported which attempt to enlarge a polyketide synthase by interpolating additional modules.

Results: We describe here the construction of tetraketide synthases in which an entire extension module from the rapamycin-producing polyketide synthase is covalently spliced between the first two extension modules of the erythromycin-producing polyketide synthase (DEBS). The extended polyketide synthases thus formed are found to catalyse the synthesis of specific tetraketide products containing an appropriate extra ketide unit. Co-expression in *Saccharopolyspora erythraea* of the extended DEBS multienzyme with multienzymes DEBS 2 and DEBS 3 leads to the formation, as expected, of novel octaketide macrolactones. In each case the predicted products are accompanied by significant amounts of unextended products, corresponding to those of the unaltered DEBS PKS. We refer to this newly observed phenomenon as 'skipping'.

Conclusions: The strategy exemplified here shows far-reaching possibilities for combinatorial engineering of polyketide natural products, as well as revealing the ability of modular polyketide synthases to 'skip' extension modules. The results also provide additional insight into the three-dimensional arrangement of modules within these giant synthases.

Keywords: Polyketide synthase; Modular; Erythromycin; Rapamycin; Module insertion; Octaketide; Skipping; *Saccharopolyspora erythraea*

1. Introduction

Complex polyketides are a large and structurally diverse class of natural products that includes many compounds possessing antibiotic or other clinically useful properties. They are each produced by stepwise chain assembly on a modular polyketide synthase (PKS). Such PKSs are giant multienzymes containing a different set or 'module' of enzyme domains to accomplish each successive cycle of polyketide chain extension [1,2]. The evident modularity of these systems, together with numerous reports that productive hybrid PKSs may be engineered, by swapping either one [3–6] or more [7–11] domains between different

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natural PKSs, has generated increasing interest in the degree to which this modularity might be exploited to make large numbers of new, potentially valuable natural products. In choosing the points of fusion in such hybrid PKSs, linker regions are normally selected from within the pronounced domain-and-linker structure of modular PKSs in solution as predicted by computer-assisted sequence alignments [12–14] and determined by limited proteolysis [15,16] of the purified multienzymes. It has also proved fruitful to create such fusions at the margin of a domain, within regions of conserved sequence [3,8]. Meanwhile, the importance has also been highlighted of intermodular transfer [10,11], of chain release [17,18], and of the action of auxiliary thioesterases [19,20], as potentially rate-limiting steps in polyketide chain synthesis. In this work, we aimed to gain further insight into the role of intermodular transfer, by inserting the DNA encoding a complete extension module, derived from the rapamycin-producing PKS of *Streptomyces hygroscopicus*, into the gene encoding the erythromycin-producing PKS of *Saccharopolyspora erythraea*, the 6-deoxyerythronolide B synthase (DEBS), to produce a molecular assembly line extended in the middle by one module. Removal of C-terminal modules was originally accomplished by re-location of the DEBS chain-terminating thioesterase [21], and almost all subsequent studies on this enzyme have exploited this strategy, which leaves the PKS structure relatively unperturbed, to select polyketide chain length and to ensure efficient release of polyketide products. Insertion of an entire module into the middle of an existing modular assembly presents a significantly greater challenge to the structural integrity and proper functioning of the resultant hybrid. Accordingly, any success in this would be particularly relevant to the prospects for combinatorial deployment of individual modules from diverse sources.

The modular PKSs are homodimers [22], with close functional links between identical modules in the paired multienzyme subunits [16,23]. We have proposed [16] that each module interacts with its partner in a configuration which is both head-to-tail and head-to-head, so that the ketosynthase (KS) and acyltransferase domains from each module constitute a tetrahedral core. The overall fold of the PKS in the preferred version of this model is a double-helical arrangement, in which the chirality of each successive module pair is identical, for the entire PKS. There is therefore no stereochemical constraint on modules being swapped, deleted or inserted to generate functional hybrid PKS assemblies.

We now report the production of novel tetraketide products by addition of a complete module into the model system DEBS1-TE derived from the erythromycin PKS of *S. erythraea* [21], a bimodular PKS that normally makes triketides; and also the production of novel octaketides, both 16-membered and 14-membered macrolactones, by addition of a complete module into the intact erythromycin PKS, a hexamodular PKS which normally makes heptaketide macrolactones only. Our results demonstrate that module-by-module assembly of natural PKS modules into novel multienzymes is a realistic procedure for combinatorial biosynthesis. The module insertion procedure is not yet efficient: the major compounds produced by the extended PKSs were the normal products of the unextended PKS, a phenomenon we have referred to as 'skipping'.

2. Results

2.1. Construction of an extended modular tetraketide synthase

The sequencing of the rapamycin (*rap*) PKS gene cluster [14,24,25] provided the genetic material for diverse PKS modules, for use as spare parts in construction of hybrid PKSs. For the present experiments, we initially chose to insert rapamycin PKS modules 2 and 5 into the well-characterised DEBS1-TE system, a truncated bimodular PKS derived from the first two extension modules of the DEBS of *S. erythraea* (Fig. 1). Both rapamycin modules 2 and

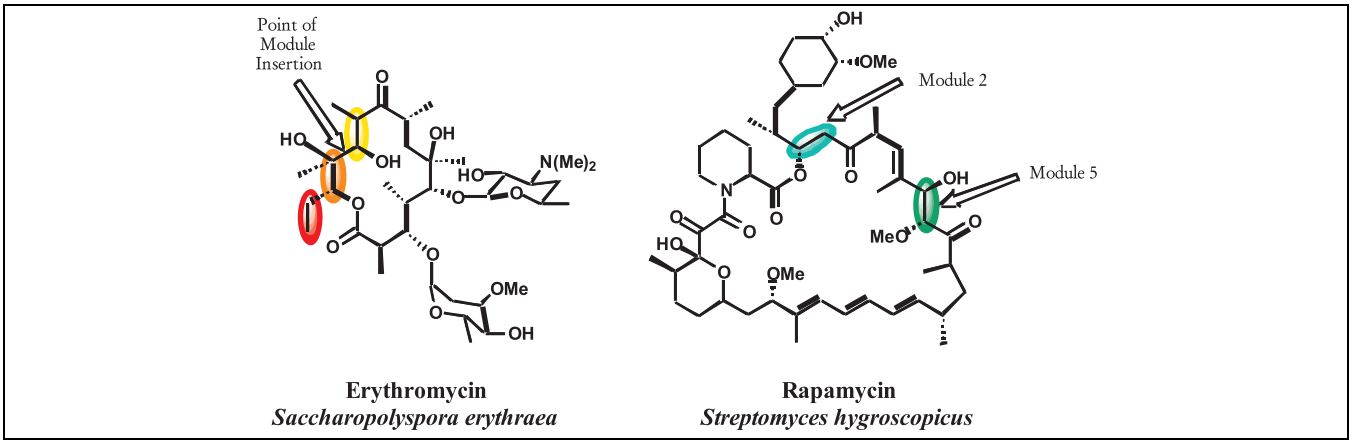

Fig. 1. The structures of erythromycin A and rapamycin. The ketide units introduced through the action of the rapamycin-producing PKS modules 2 and 5 are highlighted. Also highlighted is the site in the erythromycin macrocycle chosen for insertion of the new ketide unit.

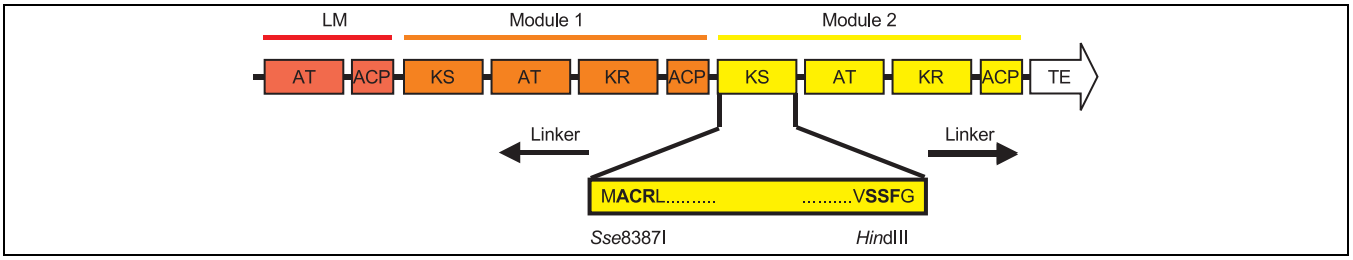

Fig. 2. Domain structure of DEBS1-TE. A variant DEBS1-TE PKS gene was engineered as a host for module insertion by introduction of a unique *Sse*8387I site and a unique *Hin*dIII site in the DNA encoding the edges of the KS domain of module 2, without alteration in the amino acid sequence of the residues specified (shown in bold type). AT, acyltransferase; ACP, acyl carrier protein; KS, β-ketoacyl synthase; KR, β-ketoacyl reductase; DH, dehydratase; ER, enoylreductase; TE, thioesterase. LM, Loading Module.

5 normally catalyse the extension of a polyketide chain by the addition of an acetate unit and stereospecific reduction to a hydroxyacyl-thioester, before transfer to the next module for subsequent rounds of chain extension. In each case, the additional module was spliced into the N-terminal flanking region of the KS domain of module 2 of DEBS, at a site not far from the inter-domain linker, and which introduced no amino acid changes in the amino acid

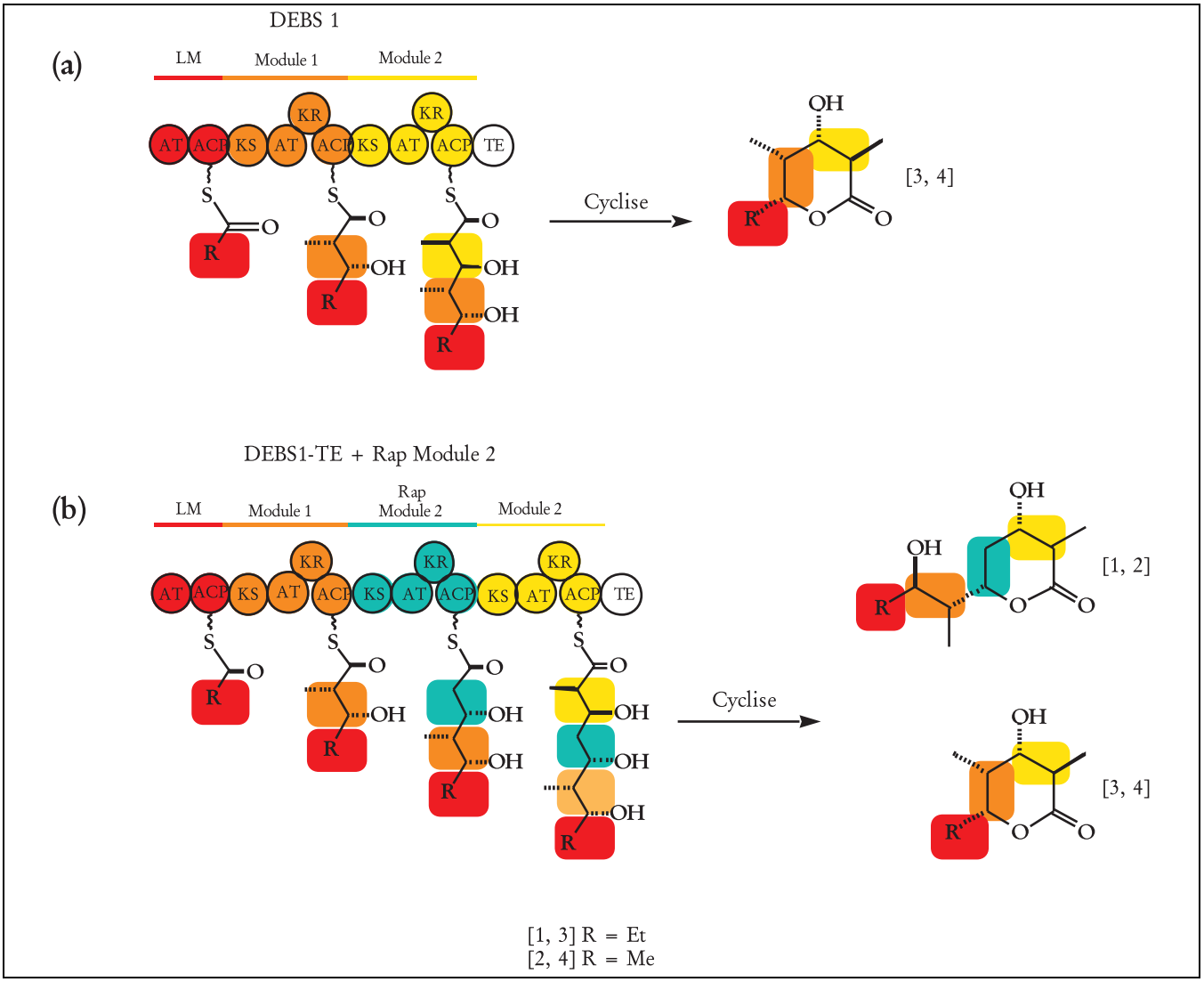

Fig. 3. Domain organisation of (a) the bimodular DEBS1-TE and (b) trimodular synthases obtained by engineered insertion of module 2 of the rapamycin-producing PKS (*rap* module 2) at the N-terminus of DEBS module 2. Orange spheres represent domains originating from DEBS module 1, yellow spheres represent domains originating from DEBS module 2, and green spheres represent domains from *rap* module 2. Also shown are the full-length products found to be produced in vivo from each multienzyme. The tetraketide synthase in (b) produced the triketide lactone **1** as a major product.

sequence (Fig. 2). Standard cloning protocols were used to obtain rapamycin module 2 as an *Sse*8387I fragment from the beginning of KS2 to the beginning of KS3; and to introduce a unique *Sse*8387I site into the beginning of the KS2 in the gene encoding DEBS1-TE. The heterologous module was then introduced in the correct orientation to yield an in-frame tetraketide synthase. This new open reading frame was cloned into the expression vector pCJR24[26] to give pCJR54. In this expression plasmid, the tetraketide synthase is placed under the *actI* promoter (P_{actI}) which is under the control of its cognate activator *actII*-ORF4.

To assess the productivity of the expanded PKS we used as hosts *S. erythraea* NRRL2338 red variant [27] and two mutant strains derived from it. *S. erythraea* JC2 [26] is a strain in which the entire *eryA* region, encoding the DEBS multienzymes, has been deleted, except for the DNA encoding the C-terminal chain-terminating thioesterase, which is retained as a region of homology for recombination. *S. erythraea* strain No. 5 [28] is deficient in deoxysugar biosynthesis and accumulates the aglycones erythronolide B and 6-deoxyerythronolide B.

Protoplast transformation of *S. erythraea* JC2 with plasmid pCJR54 followed by a single recombination event, into the thioesterase region, yielded the strain *S. erythraea* JC2/pCJR54 in which there is a tetraketide synthase located in the chromosome under P_{actI}. When grown on SM3 agar for 16 days *S. erythraea* JC2/pCJR54 produced 1–2 mg/l amounts of both the expected tetraketide products **1** and **2**, representing alternative acetate and propionate starter units, and also the triketide products **3** and **4** shown in Figs. 3 and 4. The ratio of tetraketide to triketide products was approximately 1:20, as judged by gas chromatography mass spectrometry (GC-MS) analysis. The overall yields of purified polyketide products observed are comparable to yields from a recombinant *S. erythraea* in which a chromosomal copy of DEBS1-TE is under the control of the actinorhodin *actI* promoter, i.e. 20–40 mg/l in total. The structures of the triketide products were those expected if DEBS modules 1 and 2 had been utilised, but the interpolated rapamycin module had been bypassed during chain extension.

Extraction of the agar with ethyl acetate, followed by rigorous purification, and analysis by high resolution mass spectrometry and multidimensional ^{1}H nuclear magnetic resonance spectroscopy, confirmed the structure and stereochemistry of the tetraketide **1**, with the stereochemistry of module 1 being deduced from the expected stereochem-

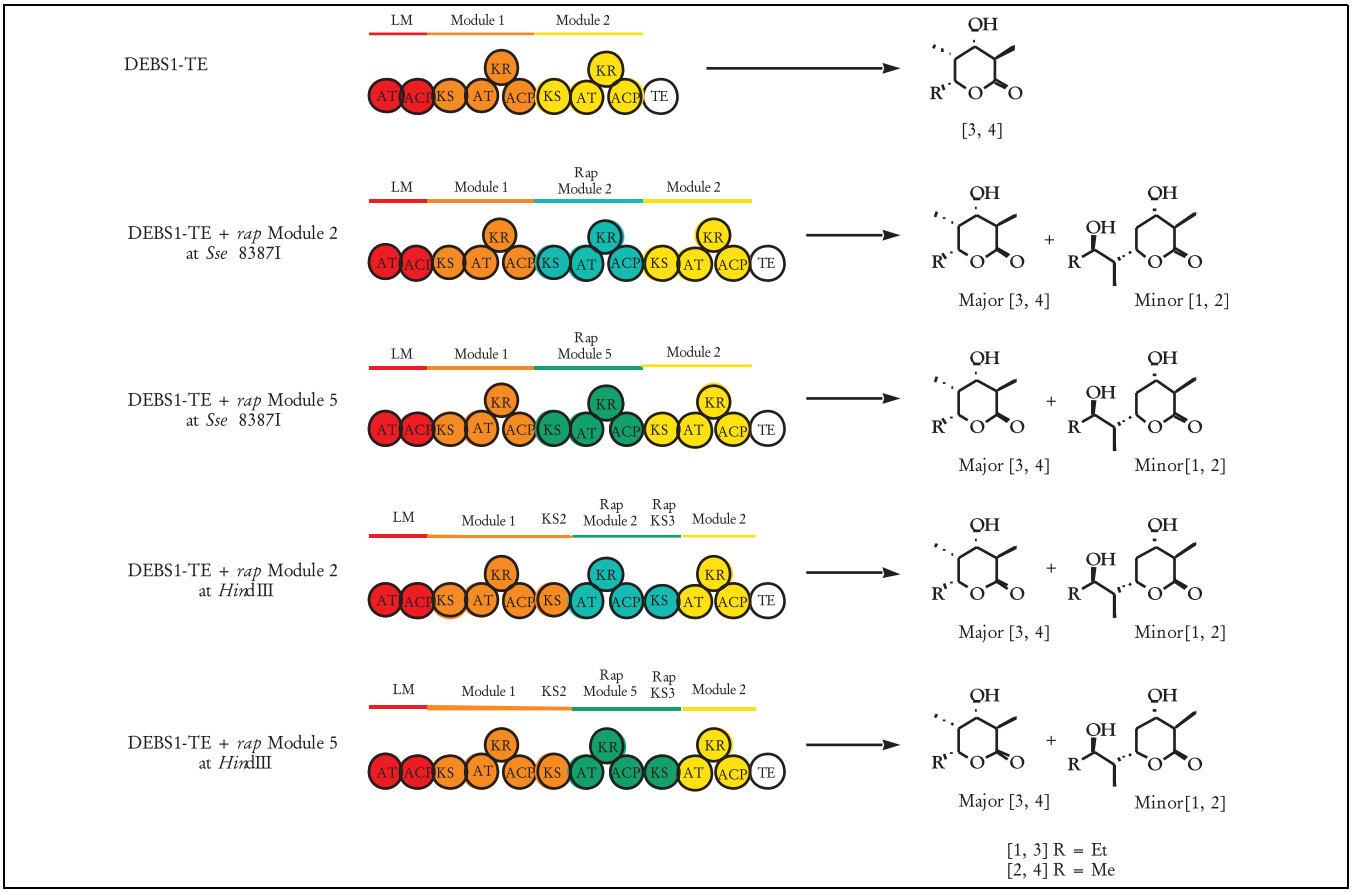

Fig. 4. Domain organisation of hybrid trimodular PKS multienzymes made in this study. The domain organisation of the bimodular PKS DEBS1-TE is shown for comparison.

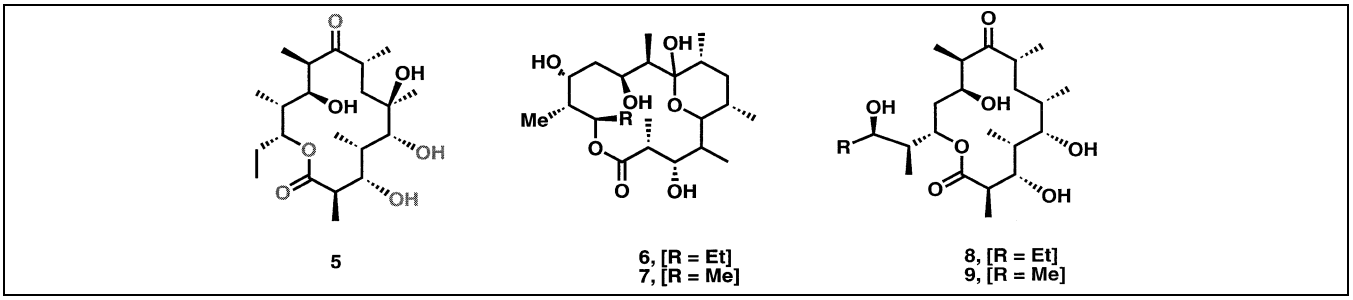

Fig. 5. Hepta- and octaketide macrolactones produced by DEBS and/or the heptamodular PKS multienzymes constructed in this work.

ical outcome of DEBS module 1. The structures of the two triketides were fully confirmed by GC-MS and LC-MS and comparison with synthetic standards.

The above experiments were repeated, except that the module inserted into DEBS1-TE was *rap* module 5, which was predicted to function exactly as for *rap* module 2. In agreement with this, the same novel compounds **1** and **2** were obtained in similar yields, and again the ratio of novel compounds to the triketide lactones **3** and **4** was 1:20.

2.2. *Construction of an octaketide synthase*

Protoplast transformation of *S. erythraea* No. 5 with the plasmid pCJR54, followed by selection for integration of the plasmid into the chromosome by homologous recombination, could in principle give one of several alternative outcomes, because integration might occur in the region of either DEBS module 1, DEBS module 2, or the thioesterase domain. Recombinants were sought in which recombination into the chromosomal copy of *ery* module 2 had occurred. Such recombination leads to a triketide synthase under the control of the resident *ery* PKS promoter, while the octaketide synthase is placed under the powerful *act* promoter and its activator. One such recombinant was identified, as confirmed by Southern analysis (data not shown) and named *S. erythraea* No. 5/pCJR54. This strain was grown in liquid culture as described in Section 5. Extraction of the culture with ethyl acetate and LC-MS analysis established that two different

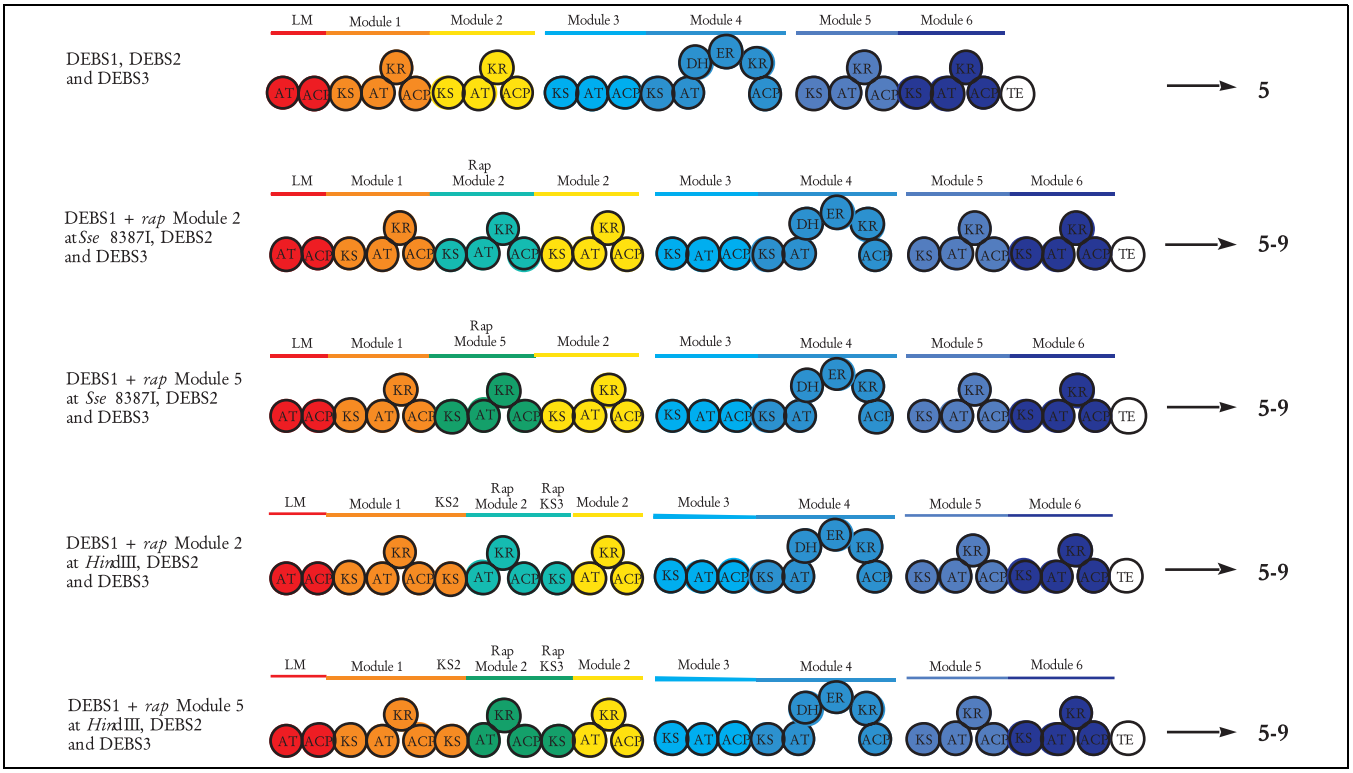

Fig. 6. Domain organisation of hybrid heptamodular PKS multienzymes made in this study. The domain organisation of the hexamodular erythromycin-producing PKS is shown for comparison.

novel compounds were present with the masses expected for each of the two predicted octaketide products (containing either acetate or propionate starter units). Erythronolide B **5** was also present, as the most abundant macrolactone, at about 10 mg/l, the same level as obtained from a strain carrying the normal DEBS genes under the control of the *actI* promoter. Three of the four novel compounds detected were isolated and their structures were determined by high resolution MS and multidimensional ^{1}H and ^{13}C nuclear resonance spectroscopy. These three compounds (Figs. 5 and 6) are the expected 16-membered macrolactones **6** and **7**, containing either a propionate or an acetate starter unit respectively. The third is a 14-membered macrolactone **8**, which is formed from the same enzyme-bound octaketide as **7**, but in which the hydroxyl at C-13 is used for cyclisation rather than the hydroxyl at C-15. The corresponding 14-membered macrolactone **9** with a propionate starter was present in relatively small amounts (less than 1 mg/l) and was not isolated. The 16-membered macrolactone products were isolated as the hemiketal forms, whereas the 14-membered product was isolated as the macrolactone. The ratio of octaketide to heptaketide products, based on isolated yields, was 1:3.5. No evidence was obtained for hydroxylation at C-6 of the 16-membered macrolactones or of the 14-membered macrolactone at C-6, the first step in further processing of 6-deoxyerythronolide B during erythromycin biosynthesis. The inability of the C-6 hydroxylase to act on macrolactone **8** indicates a narrow substrate specificity for this enzyme.

To assess whether these novel aglycones might serve as substrates for the later enzymes of the erythromycin biosynthetic pathway, protoplasts of wild-type *S. erythraea* were transformed with plasmid pCJR54. Recombinants were selected that had undergone homologous recombination into *ery* module 2, as confirmed by Southern analysis (data not shown). One such strain, *S. erythraea* WT/pCJR54, was fermented and the 16-membered macrolactone products were purified and characterised as before. The same macrolactone products **6**–**9** were again observed, and no evidence was found for the presence of glycosylated derivatives of these compounds.

The module insertion experiment was then repeated, except that the module interpolated was *rap* extension module 5, spliced into a unique *Sse*8387I site at the N-terminus of the KS2 domain in the gene encoding DEBS1-TE. Recombinants containing the corresponding octaketide synthase in *S. erythraea* NRRL2338 (red variant) and the mutant strain *S. erythraea* No. 5 derived from it, were fermented and the macrolactone products extracted and characterised. Again, the novel compounds **6**–**9** were detected together with substantial amounts of normal 14-membered macrolactone products, indicating a high level of skipping in these hybrid PKSs too (Fig. 6).

2.3. Insertion of rap module2 and rap module 5 at alternative sites within DEBS1-TE

In further experiments, the DNA for an additional module was spliced into the gene for DEBS1-TE using a unique *Hin*dIII site, engineered into the DNA for the linker region C-terminal of the KS domain of DEBS module 2 (Fig. 2). This extended PKS contains the *ery* KS2 fused to the AT domain of the interpolated rap module (which extends from rap AT2 to the end of the KS domain of *rap* module 3), and the *rap* KS3 is fused to the N-terminus of the AT domain of DEBS module 2. Another version of this extended PKS was created using *rap* module 5, likewise inserted at the *Hin*dIII site. The methods used for cloning, expression, and product analysis were mutantis mutandis exactly analogous to those given in detail in Section 5 for insertions at the *Sse*8387I site. In each case the pattern of production of tetraketides, and their skipped products, was found to be exactly the same (data not shown) as when the module was inserted before the KS2 domain (Fig. 4).

3. Discussion

3.1. Module insertion yields full-length polyketide products of the predicted structure

The successful insertion of a complete heterologous module, in covalent linkage, into the middle of a pre-existing PKS multienzyme, is one of the most dramatic illustrations so far of the modularity of these systems. Also, the full-length polyketide products proved to have exactly the predicted structure, given the nature of the inserted module. As has been frequently observed with previously engineered hybrid PKSs [5,6,11], the efficiency of production of the target compounds was lowered (to about 3–5%) compared to the production of the normal polyketide products, although as discussed below the overall productivity of the hybrids made here was comparable with that of the normal PKS. In part this loss of efficiency in obtaining the target compounds in the present case can be ascribed to the fact that the incoming module and the adjacent modules were not matched in terms of the chemistry and stereochemistry of the ketide units they insert. Rather, the modules for insertion were chosen so as to provide a chemical outcome readily distinguishable from the normal operation of the parent PKS. It is clear that the enzymes of both the interpolated *rap* module, and of the downstream *ery* module 2, will be confronted with substrates that are unnatural, and there may be a loss of efficiency at both steps.

There was no difference in the outcome when rapamycin module 5 was substituted for rapamycin module 2, despite their different native contexts within the rapamycin PKS.

This implies that differences in the efficiency of molecular recognition by each module are not the sole or even the major determining factor in the lower yield. From recombinant *S. erythraea* expressing the hybrid tetraketide synthases, cell-free extracts were found to contain polypeptides of the expected size for a trimodular PKS, and at levels somewhat reduced compared to the parent DEBS1-TE (data not shown) so a lower expression also may account for some, but again only a part, of the difference.

3.2. Insertion of modules at different sites

Further experiments were also undertaken in which the inserted rapamycin PKS module extended not from the KS domain to the ACP, but rather extended from the end of the KS domain to the end of the KS domain of the next module. In other words, the module embraced either from the AT2 domain to the KS3 domain, or from the AT5 domain to the KS6 domain, depending on which module was being used. In these cases the module was inserted in the DNA encoding the C-terminal of the *ery* KS2 domain in the DEBS1-TE, at a unique *Hin*dIII site, to give the expanded trimodular PKS. Fermentation of strains containing these constructs gave exactly the same reaction products in about the same yields, as were seen when insertion was done in the DNA encoding the N-terminus of the KS2 domain, at a unique *Sse*8387I site (Figs. 4 and 6). Although the interpretation is clouded by uncertainties over the relative expression levels of the hybrid enzymes, these results demonstrate that module insertion is not wholly dependent on a uniquely correct choice of insertion site.

3.3. The observation of skipping in extended modular PKSs

The most striking and unexpected feature of the operation of the extended PKS multienzymes constructed here was the observation, in every case, that the major products of the fermentation were the products of the parent unextended PKS. Thus the trimodular PKSs all produced triketide lactones **3** and **4** in amounts (20–40 mg/l) which are those expected of the normal DEBS1-TE triketide synthase; and erythronolide B **5** was a major product of the heptamodular PKSs, in amounts (10 mg/l) not much reduced compared to the normal PKS. We have called this new phenomenon, in which an extension module within a modular PKS multienzyme is effectively bypassed, 'skipping'. The ratio of expected to skipped products was measured at 1:20 for the triketides, and 1:3.5 for the octaketides, but these figures are based on isolated yields and the true figure is likely not different for the two systems. There is present in the octaketide-producing strains a DEBS1-TE gene under the control of the chromosomal *ery* promoter, but the effects of enzyme produced from this will be limited, perhaps marginally reducing the overall yield of macrolactone, or allowing a minor alternative pathway for production of the normal erythronolides.

At present the mechanism of the skipping phenomenon is unknown, but two broad types of mechanism can be distinguished: first, the formation of the unextended product may be dependent on the enzymatic activities of the interpolated module, which would imply that the extended PKS is correctly folded overall, but that the condensation step and/or the recruitment of the extender unit may be adversely affected. Alternatively, the formation of the unextended polyketide products might be independent of the activity of the incoming module. This might be true if the structure of the extended PKS is distorted in a major way, so that direct polyketide chain transfer between the flanking modules can occur, or if a carrier such as coenzyme A ferries the polyketide chain between them. It cannot be ruled out that the reduced yields of the expected products might have been obtained even if skipping had not occurred. We cannot at present distinguish the case where the presence of stalled polyketide chains on the PKS (because of poor activity of the hybrid enzyme) provokes skipping, from one where the possibility for skipping actively diverts polyketide chains away from the production of full-length products.

It has been previously reported that a trimodular PKS based on the first three extension modules of the erythromycin PKS synthesises in vitro not only tetraketide products but also triketide lactones **3** and **4**, the predicted products of the operation of modules 1 and 2 only [29]. The thioesterase domain linked covalently to the C-terminus of module 3 was reportedly able to cyclise the triketide acyl chain faster than the TE domain on its own. An explanation for these results, in the light of our data, is that the triketide was passed to the thioesterase via module 3, which was skipped. The term skipping has recently been used [30] to describe the operation of the pikromycin/methymycin PKS of *Streptomyces venezuelae*, which is responsible for the formation of both hexaketide and pentaketide products from a single enzyme [19]. Sherman and his colleagues have shown convincingly that under certain growth conditions the PKS polypeptide bearing the final module is expressed in a truncated form lacking the N-terminal portion of the KS domain, and that this leads to pentaketide synthesis [30]. However, it remains to be proved whether the release of pentaketide is indeed achieved by premature termination, through alternative docking of the thioesterase (located at the C-terminus of module 6) against the ACP of module 5, as proposed. Possibly, the pentaketide chain might 'skip' through module 6 to reach the thioesterase, without such major rearrangement of the PKS structure.

3.4. Implications of module insertion for structural models of modular PKSs

The results of module insertion reported here do not

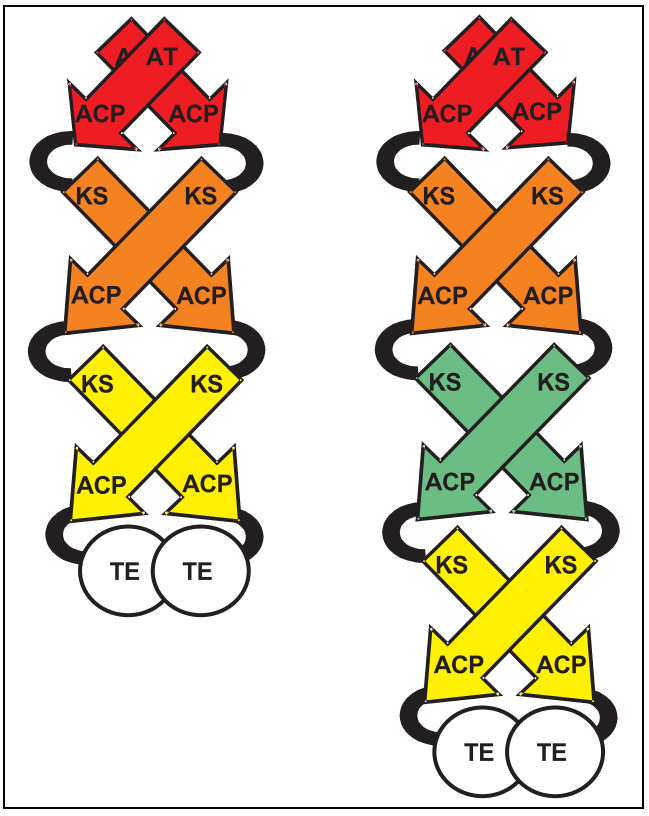

Fig. 7. Consequences of insertion of a module into a PKS multienzyme. Schematic three-dimensional representations of DEBS1-TE based on the proposed helical model [16]: consecutive module pairs are twisted in the same sense so there is no restriction on the interpolation of an additional module between two extension modules.

lend unequivocal support to any of the structural models that have been advanced for these multienzymes in the absence of high resolution structural information. However, as illustrated in Fig. 7, the tetrahedral/helical model [16], and a variation on it advocated recently [31] both predict that all module pairs have the same chirality (arbitrarily shown in Fig. 7). An additional module can therefore be readily accommodated without gross conformational changes.

4. Significance

Modular PKSs are responsible for the biosynthesis of a wide range of clinically important natural products, including antibacterial, antitumour, immunosuppressant and antiparasitic compounds. As in non-ribosomal peptide synthetases, the separate set or 'module' of enzymes that together catalyse each successive cycle of chain extension are encoded together, and are housed together in giant multienzymes, in the order in which they are used. We have demonstrated here that genetic engineering may be used to insert an entire heterologous extension module of activities into an existing modular PKS, and that the resulting hybrid catalyses the production of a full-length product with exactly the predicted structure. This ability to extend PKS assembly lines by insertion provides a new way in which the individual modules of natural PKSs can be combined in a combinatorial fashion to generate novel polyketide libraries as potential leads in drug discovery. The desired full-length products were found to be accompanied by large amounts of 'skipped' polyketide products, produced by an unknown mechanism.

5. Materials and methods

5.1. Chemical analysis

^{1}H NMR spectra were recorded at 500 MHz on a Bruker AMX-500 or DRX-500. ^{13}C NMR spectra were recorded at 125 MHz on a Bruker AMX-500. ^{1}H and ^{13}C NMR spectra were referenced internally to $CHCl_3$ (7.27 and 77.5 ppm respectively). *J* values are given in Hz. GC-MS was performed on a Finnigan MAT GCQ instrument. Analytical and preparative reverse phase high performance liquid chromatography mass spectrometry (HPLC-MS) analysis was carried on a Finnigan MAT LCQ instrument. Analyses were run using either an ES or API-MS interface and using positive–negative ion mode switching. 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.

5.2. Bacterial strains and culture conditions

Escherichia coli DH10B (Gibco BRL) was used in all standard cloning procedures and were grown in 2×TY medium [32,33]. Electrocompetent cells of DH10B were made as described previously [34]. *S. erythraea* NRRL2338 (red variant) [27] was the kind gift of Dr J.M. Weber. *S. erythraea* mutant strain JC2 (from which the erythromycin-producing PKS genes have been deleted apart from the chain-terminating thioesterase domain) has been described [26] as has *S. erythraea* mutant strain No. 5, which accumulates erythronolide B [28]. *S. erythraea* strains were routinely maintained on R2T20 [35] and TSB medium (Difco) for liquid cultures at 30°C. After ∼2 weeks on R2T20 agar at 30°C spores were harvested and stored in 10% glycerol at −80°C.

5.3. Plasmids and DNA manipulation procedures

Plasmid pCJR24-S was derived from pCJR24 [26] to eliminate an unwanted *Sse*8387I site in the polylinker by digesting with this enzyme, filling-in and religation. Plasmid pIB103 is a pCJR24-S-based plasmid containing the DEBS1-TE gene, into which a unique *Sse*8387I site (coincident with a unique *Pst*I site) and a unique *Hin*dIII site have been introduced by site-specific mutagenesis using PCR (Fig. 2). A *S. erythraea* strain housing this modified DEBS1-TE gene was shown in control experiments to produce the expected triketide lactones in similar amounts to a

strain containing DEBS1-TE. Plasmid preparation, restriction-enzyme digestion, fragment isolation and cloning were performed using standard procedures [32,33] PCR was performed using *Pwo* polymerase (Boehringer) according to the manufacturer's instructions.

5.4. Construction of plasmid pCJR54 for the hybrid synthase containing rap module 2

DNA encoding extension module 2 of the rapamycin-producing PKS [14,24] was cloned with flanking *Sse*8387I sites as follows: a fragment encoding the N-terminal region, up to a unique *Sal*I site, was amplified by PCR using the oligonucleotides 5′-CGCCGT*GTCGAC*CGTGAACGCCG-3′ (reverse) and 5′-GTATGG*CCTGCAGG*TTGCCGGGTGGGG-3′ (forward); a fragment encoding the C-terminal region up to a unique *Cel*II site was amplified by PCR using the oligonucleotides 5′-GGTAG*CCTGCAGG*CCATTCCCACG-3′ (reverse) and 5′-TCCTGGACGCGCT*GGCTGAGC*A-3′ (forward) (restriction sites are shown in italics); each of these fragments was cloned into *Sma*I-cut pUC18 (where the DNA sequence was confirmed by sequencing), then assembled by ligation with a *Sal*I–*Cel*II fragment encoding the central region of *rap* module 2. The resulting *Sse*8387I fragment housing the DNA for all of *rap* module 2 was then cloned into plasmid pIB103 which had been linearised with the same enzyme and treated with phosphatase. A plasmid containing the correct orientation of the inserted *Sse*8387I fragment was identified and was named pCJR54.

5.5. Construction of plasmid pIB107 for the hybrid synthase containing rap module 5

DNA encoding extension module 5 of the rapamycin-PKS [14,24] was cloned with flanking *Sse*8387I sites as follows: a fragment encoding the N-terminal region, up to a unique *Xho*I site, was amplified by PCR using the oligonucleotides 5′-ATGG*CCTGCAGG*CTGCCGGGTGGGGTG-3′ (forward) and 5′-CC*CTCGAG*ACCGAAGAAATACG-3′ (reverse); a fragment encoding the C-terminal region up to a unique *Xmn*I site was amplified by PCR using the oligonucleotides 5′-AG*CCTGCAGG*CCATACCCACGATCGC-3′ (reverse) and 5′-GT*GAACCGGTTC*TGGTGGCCGCGCCG-3′ (forward); each of these fragments was cloned into *Sma*I-cut pUC18 (where the DNA sequence was confirmed by sequencing), then assembled by ligation with a *Xho*I–*Xmn*I fragment encoding the central region of *rap* module 5. The resulting *Sse*8387I fragment housing the DNA for all of *rap* module 5 was then cloned into plasmid pIB103 which had been linearised with the same enzyme and treated with phosphatase. A plasmid containing the correct orientation of the inserted *Sse*8387I fragment was identified and was named plasmid pIB107.

5.6. Construction of S. erythraea strains housing either pCJR54 or pIB107

Protoplasts of *S. erythraea* strains NRRL2338, JC2, and No. 5 were transformed with pCJR54 or pIB107 according to the procedure of Yamamoto et al. [35] as adapted by Gaisser et al. [36]. Transformants were selected on solid medium containing thiostrepton (25 mg/l).

5.7. Fermentation and extraction

Frozen spore suspension (100 μl) of each plasmid-bearing *S. erythraea* NRRL2338 and No. 5 strain was used to inoculate 50 ml of SV2 seed medium [28] in a 250 ml flask containing thiostrepton (25 mg/l). This was grown for 3 days at 30°C and 250 rpm and used to inoculate seven flasks of 300 ml SM3 production medium [28] (2% v/v) which was incubated at 28°C and 250 rpm. After 7 days the fermentation broths were harvested by centrifugation and the supernatant adjusted to pH 9.5 and extracted with 2 l of ethyl acetate. *S. erythraea* JC2(pCJR54) was grown on dishes (8×250 ml) of SM3 agar containing 50 μg/ml thiostrepton. After 16 days the plates were extracted with ethyl acetate (adjusted to pH 1 with formic acid).

5.8. Isolation and purification of tetraketide metabolites

The extracts from *S. erythraea* JC2(pCJR54) were neutralised and chromatographed on a silica gel column. Appropriate fractions were combined and further purified by HPLC using a Prodigy (Phenomenex) 5μ C_{18} column (250×4.6 mm) eluted at 1 ml/min 25–80% solvent B over 20 min: solvent A – milli-Q water; solvent B – methanol. Fractions of 0.5 ml were collected and appropriate fractions combined and the solvent removed in vacuo to yield 2.5 mg of the tetraketide lactone **1**. The product was characterised by high-resolution mass spectrometry and ^{1}H NMR. ^{1}H NMR (500 MHz, $CDCl_3$) δ=4.33 (ddd, J=3.0, 5.5 and 11.5, 6-H), 3.68 (ddd, J=3.8, 9.8 and 11.5, 4-H), 3.59 (ddd, J=3.8, 5.1 and 8.1, 2′-H), 2.28 (dq, J=6.8 and 9.8, 3-H), 2.22 (ddd, J=3.0, 3.8 and 13.2, 5-H), 1.75 (ddd, J=11.5, 11.5 and 13.2, 5-H), 1.69 (ddq, J=3.8, 5.5 and 6.8, 1′-H), 1.52 (ddq, J=5.1, 7.3 and 9.8, 3′-H), 1.51 (ddq, J=7.3, 8.1 and 9.8, 3′-H), 1.34 (d, J=6.8, C(3)-Me), 1.01 (d, J=6.8, C(1′)-Me), 0.95 (t, J=7.3, 4′-H). All the data were consistent with the proposed structure and stereochemistry of **1**. The triketide lactones **3** and **4** were identified by comparison of their GC-MS and LC-MS characteristics with authentic samples.

5.9. Isolation and purification of octaketide metabolites

The extract from the putative octaketide-producing strain of *S. erythraea* No. 5 (pCJR54) was removed in vacuo to give 1.3 g of oil, which was solubilised by addition of ethyl acetate (0.4 ml), methanol (0.8 ml), 10 mM ammonium acetate (1.2 ml) and acetonitrile: 20 mM ammonium acetate (0.8 ml) (4:1 v/v). The mixture was centrifuged to remove particulate matter and subjected to preparative HPLC. Separation was achieved using a 7μ Kromasil C_8 column (60×25 mm) connected in series with a Hypersil C_{18} BDS column (150×25 mm) eluted at 20 ml/min with a gradient of 0–75% solvent B over 60 min: solvent A – milli-Q water plus 10 ml/l 1 M ammonium acetate; solvent B – 80% acetoni-

trile/water plus 20 ml/l 1 M ammonium acetate. Fractions of 10 ml were collected and the eluate was monitored at 210 nm, then split for ES-MS analysis (negative ion mode) [28]. The extraction and identification of metabolites from *S. erythraea* strains No. 5 (pIB107), NRRL2338(pCJR54) and NRRL2338(pIB107) was performed in essentially the same fashion.

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