# A hybrid modular polyketide synthase obtained by domain swapping

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**Background:** Modular polyketide synthases govern the synthesis of a number of medically important antibiotics, and there is therefore great interest in understanding how genetic manipulation may be used to produce hybrid synthases that might synthesize novel polyketides. In particular, we aimed to show whether an individual domain can be replaced by a comparable domain from a different polyketide synthase to form a functional hybrid enzyme. To simplify the analysis, we have used our previously-developed model system DEBS1-TE, consisting of the first two chain-extension modules of the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*.

**Results:** We show here that replacing the entire acyltransferase (AT) domain from module 1 of DEBS1-TE by the AT domain from module 2 of the rapamycin-producing polyketide synthase leads, as predicted, to the synthesis of two novel triketide lactones in good yield, in place of the two lactones produced by DEBS1-TE. Both of the novel products specifically lack a methyl group at C-4 of the lactone ring.

**Conclusions:** Although the AT domain is a core structural domain of a modular polyketide synthase, it has been swapped to generate a truly hybrid multienzyme with a rationally altered specificity of chain extension. Identical manipulations carried out on known polyketide antibiotics might therefore generate families of potentially useful analogues that are inaccessible by chemical synthesis. These results also encourage the belief that other domains may be similarly swapped.

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**Key words:** acyltransferase, erythromycin, polyketide synthase, *Saccharopolyspora erythraea*, rapamycin

Received: **19 Aug 1996**
Accepted: **10 Sept 1996**

**Chemistry & Biology** October 1996, **3**:833–839

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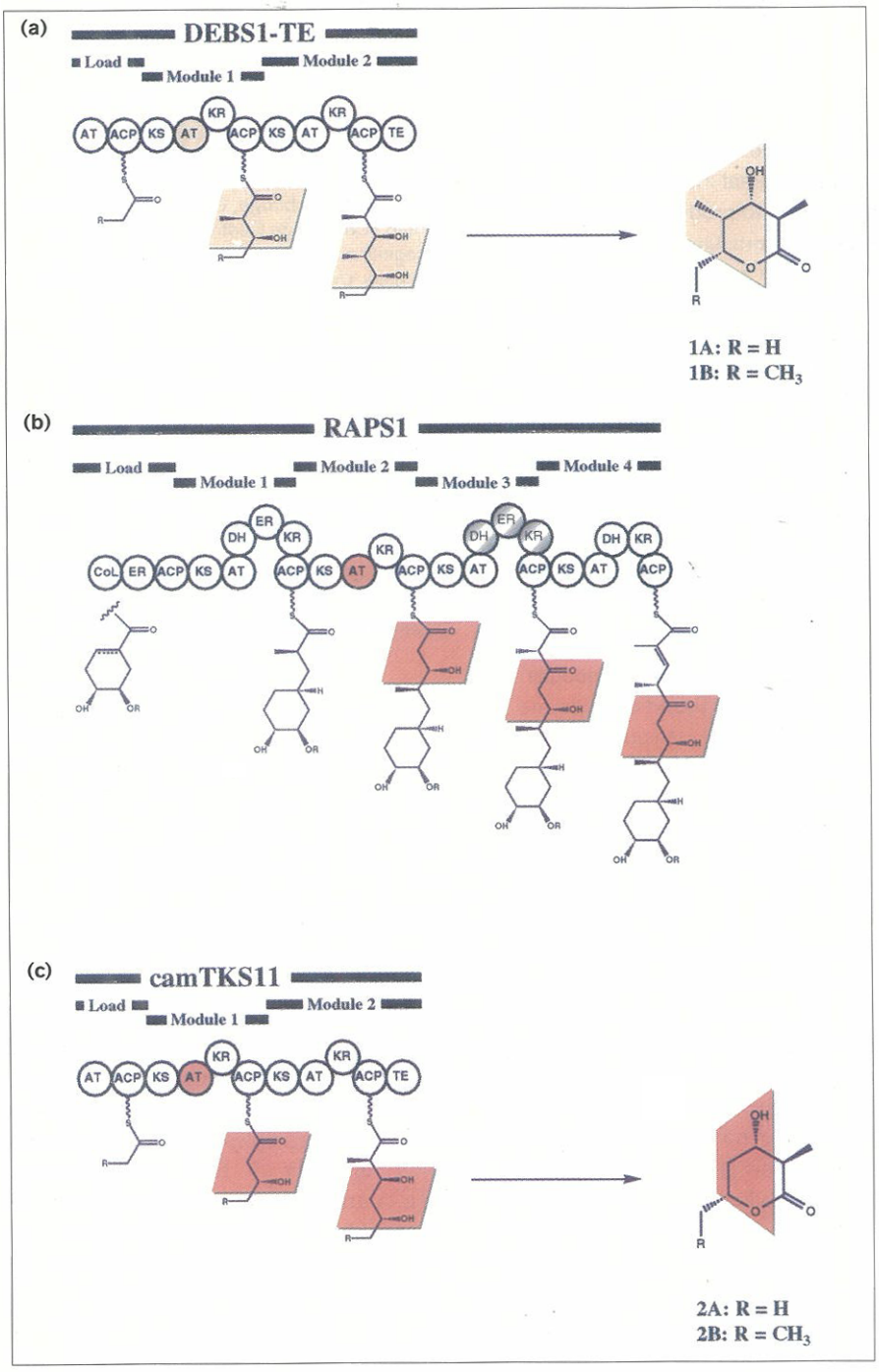
## Introduction

Complex polyketides are natural products, found predominantly in *Streptomyces* and related filamentous bacteria, that exhibit an impressive range of antibiotic, anticancer, anti-parasite and immunosuppressant activities. They are synthesized by a common pathway, in which units derived from acetate, propionate and (occasionally) butyrate 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 each PKS controls the number and type of units added, and the extent and stereochemistry of reduction at each cycle. In addition, the product of the PKS is frequently acted upon by regiospecific glycosylases, methyltransferases and oxidative enzymes, to produce still greater diversity.

Complex reduced polyketides such as the macrolides and polyethers are synthesized in bacteria on multifunctional PKSs that contain a separate set, or module, of enzymatic activities for every round of chain extension [3–7]. For example, the clinically-important antibiotic erythromycin A is derived from propionyl-CoA and six molecules of (2S)-methylmalonyl-CoA [8], through the sequential action of six such sets of enzymes housed in the three multienzyme polypeptides [9] of 6-deoxyerythronolide B synthase (DEBS) (Fig. 1). The most remarkable example so far studied is the PKS for the immunosuppressant rapamycin from *Streptomyces hygroscopicus*, which contains 14 modules housed in only 3 huge multienzyme polypeptides [10]. In contrast to the modular type I PKS multienzymes, bacterial aromatic polyketides are synthesized by type II PKSs, comprising a single set of enzymes housed in separate proteins [11–14].

Considerable interest has developed in the possibility of generating hybrid polyketides by suitable combination of activities from different natural polyketide synthases. In pioneering experiments, Strohl and colleagues [15,16] showed that when portions of the *act* biosynthetic gene cluster of *Streptomyces coelicolor*, encoding the type II PKS, were introduced into anthracycline-producing strains of *Streptomyces galileus*, novel polyketide metabolites were produced, whose structures demonstrated that productive hybrid type II PKSs were indeed formed. A large number of such experiments have since been systematically

**Figure 1**




Modular PKSs and their products. **(a)** Domain organization of the truncated triketide lactone synthase derived from DEBS. Each protein module contains an acyl carrier protein (ACP), an acyltransferase (AT), a β-ketoacyl-ACP synthase (KS), and a β-ketoacyl-ACP reductase (KR). The thioesterase/cyclase (TE), which normally cyclizes the full-length chain to 6-deoxyerythronolide B, is fused to the carboxyl terminus of DEBS 1. Cyclization at this stage is predicted to produce the triketide lactones **1A** and **1B**. The acyltransferase domain of module 1 (AT1), which is specific for methylmalonyl-CoA, is highlighted together with that part of the growing chain directly affected by the activities of module 1. **(b)** Domain organization of RAPS1, the first multienzyme component of the rapamycin-producing PKS [10,28]. Certain modules contain enoylreductase (ER) and dehydratase (DH) domains in addition to those found in the DEBS proteins. The acyltransferase domain of module 2 of the rap PKS (AT2), which is specific for malonyl-CoA, is highlighted together with that part of the growing chain directly affected by the activities of module 2. **(c)** Domain organization of the hybrid triketide lactone synthase TKScam11, constructed by replacing almost the entire AT1 domain of DEBS1-TE by rap AT2, together with the predicted products of the hybrid enzyme (**2A** and **2B**).

carried out in other laboratories [17–21] leading to the formulation of 'design rules' for production of novel aromatic polyketides [21], and to a better understanding of the role of individual components of the PKS. However, by no means every combination is productive [21], and the reasons for this remain obscure. To perform the counterpart of such 'mix and match' experiments with a modular PKS and study the design rules for these enzymes would require genetic engineering to replace an individual domain of the multienzyme by an analogous domain from another type I PKS (or fatty acid synthase) that might confer an altered specificity. We have investigated whether this is possible using our previously described model system [22] in which a chain-terminating cyclase domain (TE) has been fused to the carboxyl terminus of DEBS1, the multienzyme that catalyzes the

first two rounds of polyketide chain extension in erythromycin biosynthesis. The chimaeric DEBS1-TE was shown to synthesize efficiently the triketide lactone **1B** in *Saccharopolyspora erythraea* [22] and both **1A** and **1B** in *S. coelicolor* [23,24]. This approach has been successfully used to produce other chain lengths by truncation of DEBS [25].

We chose to replace the acyltransferase (AT) domain within module 1 of DEBS1-TE, the domain responsible for incorporation of a methylmalonyl extender unit during the first cycle of polyketide chain extension (Fig. 1). Such AT domains are present in every module and are believed to form part of the structural core of a modular PKS [26,27]. They are highly homologous as judged from sequence comparisons [28], but within this overall homology there are discernible sequence differences between those AT domains that recruit an acetate unit from malonyl-CoA and those AT domains that recruit a propionate unit from methylmalonyl-CoA (Fig. 2) [29]. We report here the results of specific replacement of the entire propionate-specific AT1 domain in DEBS1-TE with its acetate-specific counterpart from module 2 of the rapamycin-producing PKS (Fig. 1), and characterization of the products of the resulting hybrid modular PKS.

## Results and discussion

### Construction of the gene encoding the hybrid triketide synthase TKScam11

The starting point for construction of the chimaeric gene was a pT7-7-based plasmid housing the entire open reading frame for the DEBS1-TE gene. There are only two *Stu*I restriction sites within this gene, which flank the target DNA encoding the AT1 domain. The cloning strategy, as detailed in Materials and methods, was therefore to exchange the *Stu*I fragment for a new fragment into which the desired changes to the AT domain had been introduced (Fig. 3). In plasmid pMO5 the *Stu*I fragment consists of, first, a PCR-generated *Stu*I–*Bal*I fragment, which extends to just inside the amino-terminal end of AT1 and introduces a unique *Bal*I site without affecting the amino acid sequence (Fig. 2); and second, a PCR-generated fragment flanked by *Bal*I and *Avr*II sites on one end and a *Stu*I site at the other, and encoding the extreme carboxyl terminus of AT1. The DNA encoding the new AT domain derived from rapamycin module 2 is excised from plasmid pMO2 and ligated into pMO5 as a *Bal*I–*Avr*II fragment, to produce plasmid pD1-$AT_2$, encoding a full-length, heterologous AT domain (Fig. 3). All PCR products were fully sequenced to check their integrity.

The vector used for expression of the hybrid gene in *S. coelicolor* was based on the useful bifunctional SCP2*-based low copy number *S. coelicolor* plasmid pRM5, developed by Khosla, Hopwood and colleagues [17], in which genes are placed under the control of the *actI* promoter. The modified plasmid contains unique *Nde*I and *Xba*I sites downstream of the *actI* promoter, into which the altered *PKS* gene, encoding the hybrid PKS (TKScam11) was conveniently cloned as an *Nde*I–*Xba*I fragment (Fig. 3). The resulting expression plasmid pRM-AT2 was transformed into *S. coelicolor* CH999, a strain lacking the actinorhodin biosynthetic genes [17].

### Polyketide production *in vivo* by the hybrid polyketide synthase TKScam11

Previously, an engineered strain of *S. coelicolor* containing the DEBS1-TE multienzyme has been shown to produce a mixture of triketide lactones **1A** and **1B** (Fig. 1), with

**Figure 2**

Alignment of the sequences of acyltransferase domains. The AT1 of DEBS1 is aligned with AT2 of the rapamycin-producing PKS. Identical residues are shown in red. Also shown are the divergent sequence motifs identified for acetate-specific AT domains (blue) and for propionate-specific AT domains (yellow). The positions of the newly introduced restriction sites for *Bal*I and *Avr*II are indicated.

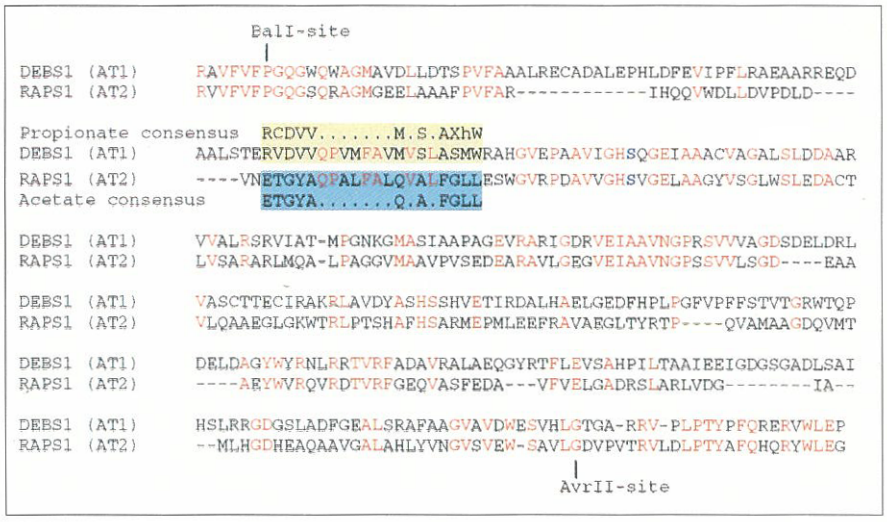
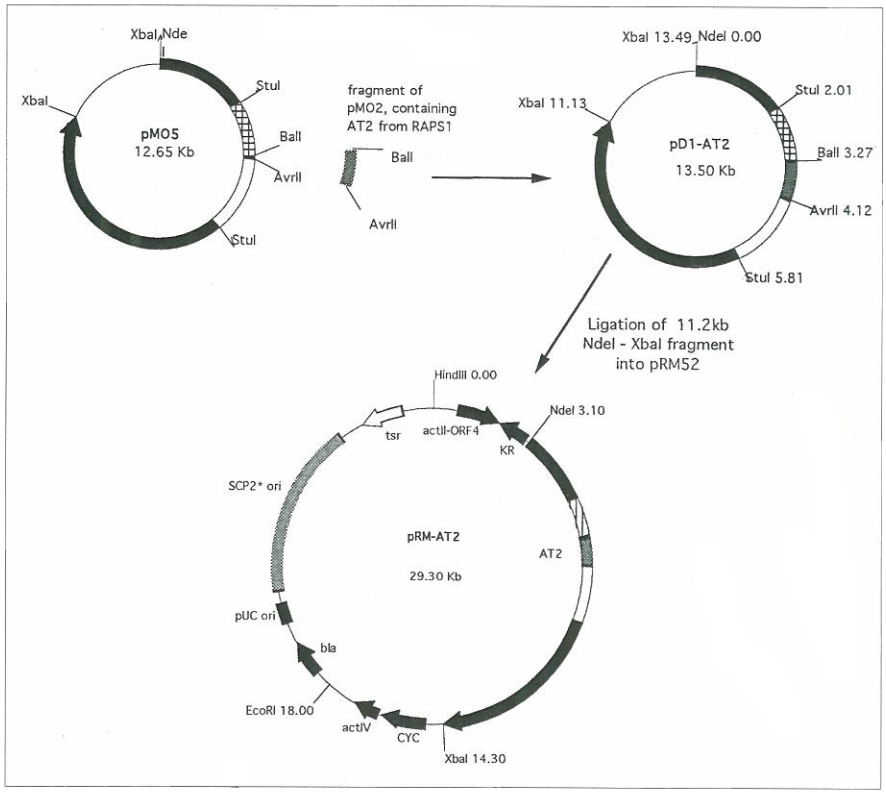


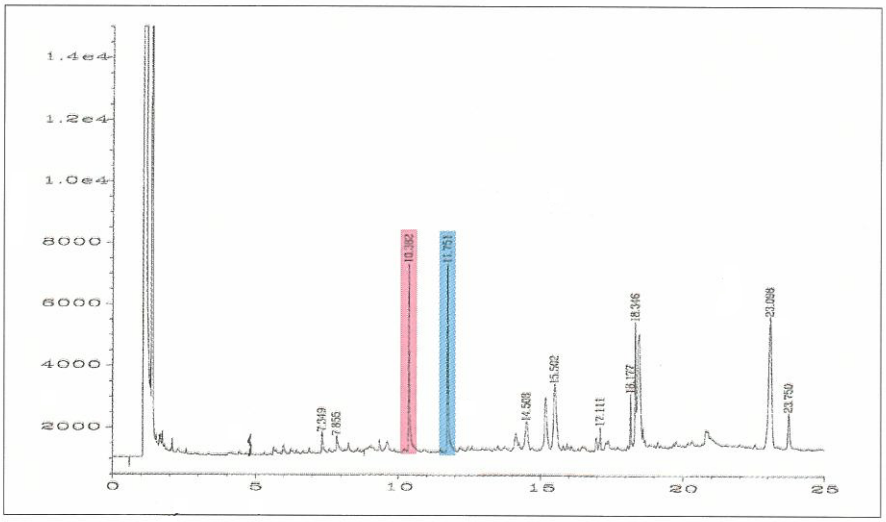
**Figure 3**




Construction of an expression plasmid containing the chimaeric polyketide synthase gene. Plasmid pMO5 is a pT7-based plasmid in which the complete open reading frame for DEBS1-TE is shown as an *NdeI–XbaI* fragment. The *StuI-BalI* fragment and the *AvrII–StuI* fragments encode the regions flanking the AT1 domain. The insertion of the *BalI–AvrII* fragment containing the AT2 domain of the rap PKS yields plasmid pD1-$AT_2$, containing the desired chimaeric gene. The gene is then subcloned into the SCP2*-derived vector pRM52 to produce the expression plasmid pRM-AT2.

**1A** being the major product [23]. For reasons that remain unclear, DEBS1 alone, when overproduced using the identical system in *S. coelicolor*, is reported by others to produce **1B** only [30]. In the present study, gas chromatography (GC) analysis of extracts from the fermentation broth of *S. coelicolor* expressing the hybrid multienzyme TKScam11 showed the complete absence of peaks corresponding either to **1A** or **1B**. In their place, two new peaks were observed (Fig. 4) which on further analysis were shown to correspond to the novel lactones **2A** and

**Figure 4**




GC analysis of polyketide products of TKScam11. The retention times for the lactones under the conditions used are as follows: 11.85 min (**1A**); 12.90 min (**1B**); 10.38 min (**2A**) (magenta); and 11.75 min (cyan) (**2B**). The ordinate shows intensity of signal in arbitrary units.

**2B** (Fig. 1), formed in roughly equal amount, and arising from the use of, respectively, acetate or propionate as a starter unit for the PKS [31,32]. The identity of the purified novel lactones was fully established by high resolution electrospray mass spectrometry, and by $^{1}H$ and $^{13}C$ NMR. The clear conclusion from these data is that the domain swap has successfully (and completely) altered the nature of the extension unit recruited by AT1 of the synthase, from a propionate to an acetate unit. There is no evidence, under the conditions used, for the formation of other diastereoisomers of **2A** and **2B**, so it appears that other events on the synthase catalyzed by module 1, particularly the reduction of the β-ketoacyl group, are not influenced by the engineered change. Similarly, module 2 has correctly processed the altered chain. The total yield of triketide lactone is also not greatly altered compared to that from DEBS1-TE in *S. coelicolor* under the same conditions [23].

As indicated in Figure 1, the normal context of the AT domain of module 2 is within the four-module multienzyme subunit RAPS1 of the rapamycin-producing polyketide synthase [10,28]. Module 2 of RAPS1 accomplishes the incorporation of an acetate unit and its reduction to a hydroxyacyl thioester, and this made it a reasonable choice to replace the AT1 of DEBS1-TE, which incorporates a propionate unit and likewise reduces it to a hydroxyacyl thioester. The success of this domain swap does not allow us to claim that any AT domain can be replaced by any other, since other pairs of AT domains may have a normal context which is less favourably matched. However, given the relatively high degree of sequence identity between AT domains from different modular PKSs, this result does hold out the promise that any complex polyketide in which propionate units occur might be engineered in an analogous fashion, to create a derivative specifically lacking one or more branching methyl groups. If this approach can indeed be generalized, it will conveniently make available derivatives of known antibiotics that would be hard to obtain by chemical synthesis. If the approach of domain swapping can also be extended to the other constituent domains of the PKS, such as the ketosynthases and ketoreductases, then the number of novel modified polyketides brought within reach would be very large indeed.

## Significance

**The modular type I polyketide synthases of *Streptomyces* and related Gram-positive bacteria constitute a unique family of carbon chain-forming multienzymes responsible for the biosynthesis of a large number of structurally diverse natural products, including antibiotics and immunosuppressants.**

**We have recently introduced a method of specifying the length of polyketide chain released from a modular polyketide synthase, and illustrated it by genetic manipulation to relocate the cyclase/thioesterase domain from DEBS3 to the carboxyl terminus of DEBS1. Strains of *S. erythraea* [22] and *S. coelicolor* [23,24] bearing the fused DEBS1-thioesterase efficiently accumulate the anticipated triketide lactones.**

**We have now shown that another type of PKS domain can function in a different context; the methylmalonyl-CoA:ACP acyltransferase domain from within extension module 1 of the DEBS1-TE has been wholly replaced by an acyltransferase domain derived from module 2 of the rapamycin-producing polyketide synthase, without loss of function. The newly-introduced domain is specific for malonyl-CoA, and we have found that the chimaeric PKS catalyzes, as predicted, insertion of an acetate unit into the triketide lactone product in place of one of the propionate units. The ability to swap domains between different modular polyketide synthases to form productive hybrids is a key step towards using these multienzymes to generate novel polyketides of potential utility as lead compounds in drug discovery.**

## Materials and methods

*Strains and plasmids*

*E. coli* TG1recO was maintained on M9 minimal medium [33] supplemented with thiamine. *S. coelicolor* CH999 was maintained on solid tap water medium [34]. SCP2*-based plasmids were selected for using thiostrepton, the kind gift of E.J. Lucania (Squibb).

*DNA manipulations*

Routine cloning and transformation procedures were as previously described for *E. coli* [33] and for *Streptomyces* [35]. PCR was carried out by the method of Saiki *et al.* [36].

*Construction of plasmid pRM52*

Plasmid pRM52 is a derivative of plasmid pRM5 [17]. Plasmid pRM5 was first linearized by digestion with *Nde*I, end-repaired and then religated to produce pRM51. pRM51 was cut with *Pac*I and *Nsi*I and the large *Pac*I–*Nsi*I fragment was isolated and ligated to a short double-stranded oligonucleotide linker containing an *Nde*I site and constructed from the synthetic oligonucleotides 5'-TAAGGAGGACACATATGCA-3' and 5'-TAATTCCTCCTGTGTAT-3', which were annealed together. The ligation mixture was transformed into *E. coli* TG1recO and isolated colonies were screened for their plasmid content. The desired plasmid (19.6 kb) was identified by its restriction map, and designated pRM52.

*Construction of expression plasmid pRM-AT2*

This was constructed in several stages, as follows. The ~1.3 kb DNA segment of the *eryAI* gene of *S. erythraea* extending from nucleotide 1 948 to nucleotide 3 273 of *eryAI* [4] was amplified by PCR using as primers the synthetic oligonucleotides 5'-CATGCTCGAGCTCTCCTG-GGAAGT-3' and 5'-CAACCCTGGCCAGGGAAGACGAAGACGG-3', and the plasmid pNTEP2 as template. Plasmid pNTEP2 is a pT7-7-based plasmid from which the entire open reading frame for the DEBS1-TE gene can be excised as an *Nde*I–*Xba*I fragment, and the details of its construction will be given elsewhere. The PCR product was end-repaired and ligated into *Sma*I-digested, phosphatase-treated plasmid pUC18, and used to transform *E. coli* TG1recO. The desired plasmid (pMO1) has the *Stu*I site adjacent to the *Hin*dIII site in the polylinker. The 0.85 kb segment of the *rapA* gene of *S. hygroscopicus*, extending from nucleotide 11 392 to nucleotide 12 235 of *rapA* [10], was amplified by PCR using the oligonucleotides 5'-TTCCCTGGCCAGGGGTCGCAGC-GTG-3' and 5'-CACCTAGGACCGCGGACCACTCGAC-3' as primers,

and the DNA from the recombinant bacteriophage λ-1C [10] as the template. The PCR product was end-repaired and ligated into *Sma*I-cut pUC18, and transformed into *E. coli* TG1recO. The desired plasmid (pMO2) was identified by its restriction pattern. The 1.7 kb DNA segment of the *eryAI* gene of *S. erythraea* extending from nucleotide 4 128 to nucleotide 5 928 of *eryAI* [4] was amplified by PCR using the synthetic oligonucleotides 5'-TGGCCAGGGAG-TCGGTGCACCTAGGCA-3' and 5'-GCCGACAGCGAGTCGA-CGCCGAGTT-3' as primers, and plasmid pNTEP2 as template. The PCR product was end-repaired and ligated into *Sma*I-cut pUC18, and the ligation mixture was transformed into *E. coli* TG1recO. The desired plasmid (pMO3) has *Bal*I and *Avr*II sites adjacent to the *Hin*dIII site of the polylinker. Plasmid pMO1 was digested with *Hin*dIII and *Bal*I and the insert was ligated into plasmid pMO3 which had been digested with *Hin*dIII and *Bal*I, to produce plasmid pMO4. Plasmid pMO4 was then digested with *Stu*I and the 3.0-kb insert was used to replace the *Stu*I fragment present in plasmid pNTEP2, to create plasmid pMO5 (Fig. 3). Plasmid pMO2 was digested with *Bal*I and *Avr*II and the insert was ligated into plasmid pMO5 which had been cut with the same two enzymes to produce plasmid pD1-$AT_2$ (Fig. 3). The hybrid PKS gene was excised from pD1-$AT_2$ as an *Nde*I–*Xba*I fragment and ligated into pRM52 which had been similarly cut with *Nde*I and *Xba*I, to produce the expression plasmid pRM-AT2 (Fig. 3).

### *Purification of (2S,3R,5R)-2-methyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone and (2S,3R,5R)-2-methyl-3,5-dihydroxy-*n*-hexanoic acid δ-lactone*

*S. coelicolor* (pRM-AT2) was grown in 3 x 500 ml YEME medium (without sucrose) for 5 days in the presence of thiostrepton (10 mg $l^{-1}$). The medium was filtered and extracted with 3 volumes of ethyl acetate, yielding 220 mg of crude product. This was absorbed onto silica (0.5 g) and partially purified by flash column chromatography (15 x 1 cm, diethyl ether). Fractions containing triketide lactones were pooled and purified by HPLC (Beckman Ultrasphere ODS 5μ, 10.0 x 250 mm, eluted isocratically at 2 ml $min^{-1}$ with 25 % MeOH:75 % $H_2O$ for 5 min followed by a linear gradient to 45 % MeOH:55 % $H_2O$ over 25 min. (2*S*,3*R*,5*R*)-2-methyl-3,5-dihydroxy-*n*-hexanoic acid δ-lactone (**2A**) has a retention time of 11 min, and (2*S*,3*R*,5*R*)-2-methyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**2B**) has a retention time of 18 min. The yield of purified **2A** was 1.5 mg $l^{-1}$, and that of **2B** was 2.0 mg $l^{-1}$.

### *Characterization of (2S, 3R, 5R)-2-methyl-3,5-dihydroxy-*n*-hexanoic acid δ-lactone (**2A**)*

$^1$H NMR ($CDCl_3$, 400 MHz) δH 4.36 (1H, dqd, J = 11.9, 6.6, 3.1 Hz, H-5), 3.75 (1H, ddd, J = 11.3, 9.9, 3.7 Hz, H-3), 2.32 (1H, dq, J = 9.9, 6.8 Hz, H-2), 2.20 (1H, ddd, J = 13.3, 3.7, 3.1 Hz, H-$4_{eq}$), 1.65 (1H, ddd, J = 13.3, 11.9, 11.3 Hz, H-$4_{ax}$), 1.40 (3H, d, J = 6.6 Hz, $CH_3$-2'), 1.40 (3H, d, J = 6.6 Hz, $CH_3$-6) ppm. High-resolution electrospray mass spectrometry (HRMS) calc'd for $C_7H_{13}O_3$: 145.08647, found $(M+H)^+$ 145.08728 (+5.60 ppm).

### *Characterization of (2S, 3R, 5R)-2-methyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**2B**)*

$^1$H NMR ($CDCl_3$, 400 MHz) δH 4.18 (1H, dtd, J = 11.8, 6.1, 2.9 Hz, H-5), 3.75 (1H, ddd, J = 11.0, 10.0, 4.0 Hz, H-3), 2.35 (1H, dq, J = 10.0, 7.0 Hz, H-2), 2.20 (1H, ddd, J = 13.3, 4.0, 2.9 Hz, H-$4_{eq}$), 1.6–1.8 (3H, m, 2xH-6, H-$4_{ax}$), 1.41 (3H, d, J = 7.0 Hz, $CH_3$-2'), 1.01 (3H, t, J = 7.5 Hz, $CH_3$-7) ppm. $^{13}$C NMR ($CDCl_3$, 100 MHz) δC 173.3 (C-1), 77.7 (C-5), 70.4 (C-3), 45.1 (C-2), 37.7 (C-4), 28.8 (C-6), 13.5 (C-2'), 9.1 (C-7) ppm. HRMS calc'd for $C_8H_{15}O_3$: 159.10212, found $(M+H)^+$ 159.10361 (+9.37 ppm).

Structural assignments were obtained for (2*S*,3*R*,5*R*)-2-methyl-3,5-dihydroxy-*n*-hexanoic acid δ-lactone (compound **2A**) and for (2*S*,3*R*,5*R*)-2-methyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (compound **2B**) using correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum coherence (HMQC) and 2D NMR spectroscopy (data not shown). Vicinal coupling constants for ring protons confirmed the assigned stereochemistry and were within 0.5 Hz of those predicted for molecular models minimized using the MM2 force-field [37] with MacroModel (version 5.5) [38]. Coupling constants were calculated using the algorithm of Haasnoot *et al.* [39].

## Acknowledgements

We thank Professors C. Khosla and D.A. Hopwood for the kind gift of *S. coelicolor* CH999 containing plasmid pRM5. This work was supported by grants from the Joint 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. P.F.L. and J.S. are members of the Cambridge Centre for Molecular Recognition.

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