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Knowledge-based design of bimodular and trimodular polyketide synthases based on domain and module swaps: a route to simple statin analogues

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Background: Polyketides are structurally diverse natural products that have a range of medically useful activities. Nonaromatic bacterial polyketides are synthesised on modular polyketide synthase (PKS) multienzymes, in which each cycle of chain extension requires a different 'module' of enzymatic activities. Attempts to design and construct modular PKSs that synthesise specified novel polyketides provide a particularly stringent test of our understanding of PKS structure and function.

Results: We have constructed bimodular and trimodular PKSs based on DEBS1–TE, a derivative of the erythromycin PKS that contains only modules 1 and 2 and a thioesterase (TE), by substituting multiple domains with appropriate counterparts derived from the rapamycin PKS. Hybrid PKSs were obtained that synthesised the predicted target triketide lactones, which are simple analogues of cholesterol-lowering statins. In constructing intermodular fusions, whether between modules in the same or in different proteins, it was found advantageous to preserve intact the acyl carrier protein–ketosynthase (ACP–KS) didomain that spans the junction between successive modules.

Conclusions: Relatively simple considerations govern the construction of functional hybrid PKSs. Fusion sites should be chosen either in the surface-accessible linker regions between enzymatic domains, as previously revealed, or just inside the conserved margins of domains. The interaction of an ACP domain with the adjacent KS domain, whether on the same polyketide or not, is of particular importance, both through conservation of appropriate protein–protein interactions, and through optimising molecular recognition of the altered polyketide chain in the key transfer of the acyl chain from the ACP of one module to the KS of the downstream module.

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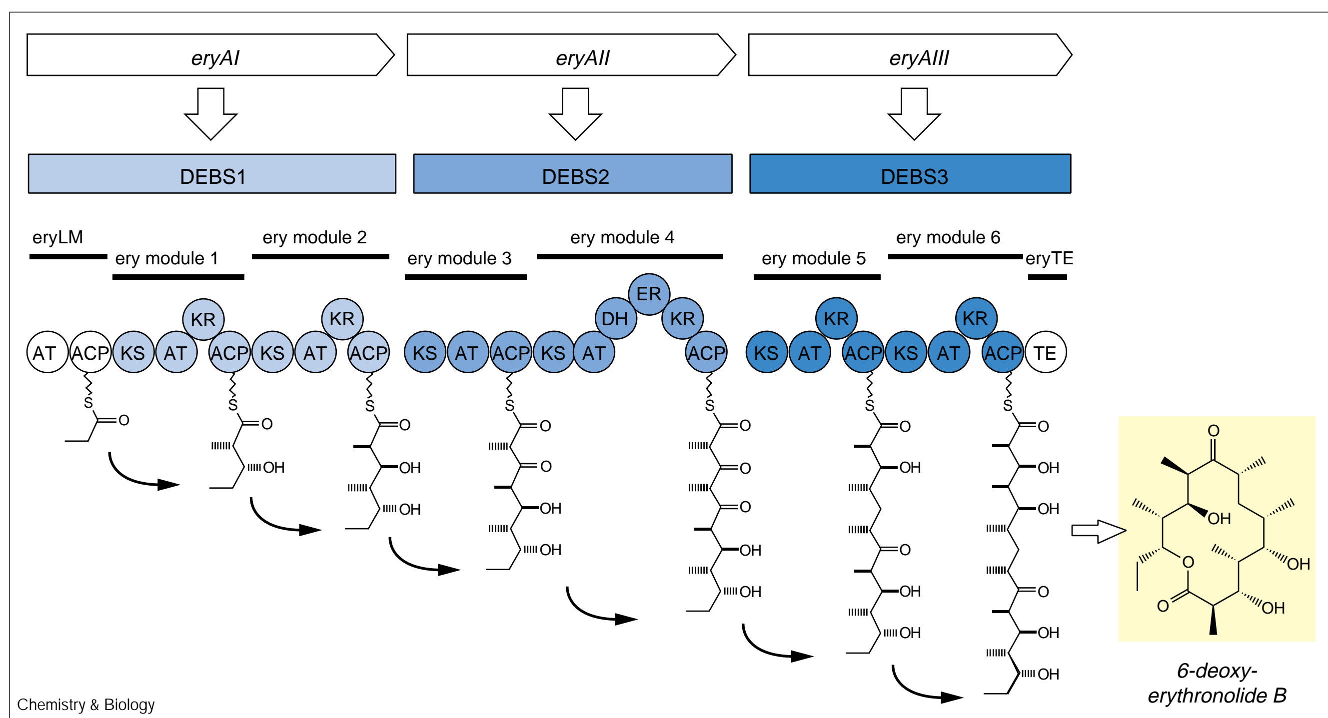
Introduction

Macrocyclic polyketides are a large group of natural products produced principally by actinomycetes using modular polyketide synthases (PKSs), which are giant multienzymes [1–8] housing sets or 'modules' of enzymatic activities, each module being required to catalyse a different cycle of polyketide chain extension. The erythromycin-producing PKS (6-deoxyerythronolide B synthase, DEBS), for example, contains six extension modules distributed among three dimeric multienzymes DEBS1, DEBS2 and DEBS3 (Figure 1) [9].

A convenient model system for studying modular PKSs has been engineered by relocating the DEBS chain-terminating thioesterase (TE) to the carboxyl terminus of the DEBS1 multienzyme [10,11]. The resulting bimodular PKS (DEBS1–TE) catalyses the production of triketide lactones both *in vivo* and *in vitro* [10–13]. The DEBS1–TE model system has been used to demonstrate that interspecies modular PKSs are functional: transplantating a malonyl-CoA-specific acyltransferase (AT) domain from extension module 2 of the rapamycin PKS to replace the methylmalonyl-CoA-specific AT domain of extension module 1 of DEBS gave rise to the expected altered triketide lactones [14]. Numerous further examples of such AT swaps have since been reported [15], as well as substitutions of reductive activities [16]. All these hybrid enzyme constructions have been guided by the results of extensive mapping,

Figure 1



The erythromycin-producing polyketide synthase (PKS); primary organisation of the genes and their corresponding protein domains. There are six chain extension modules, each beginning with a KS domain, that are responsible for adding the six successive C_3 units. The loading module (LM) specifies propionate as the starter unit. The completed chain is released as a macrolactone by the thioesterase (TE) domain. AT, acyltransferase; ACP, acyl carrier protein; KS, ketosynthase; KR, β-ketoacyl reductase; DH, dehydratase; ER, enoylreductase.

using limited proteolysis, of domain and linker boundaries of the DEBS multienzymes [17,18]. The results have tended to confirm the importance of making fusions at or near such domain boundaries or in the linker regions. These partial successes already raise the hope that libraries of bioactive products might be produced by such methods [19–22]. In this paper, we examine how our current knowledge of the structure and function of type I PKSs may be used to design and construct a modular polyketide synthase to catalyse the synthesis of specified novel products. This is a stringent test of our understanding, and success would herald a potentially important role for these enzymes in chiral synthesis.

Our biosynthetic target is a group of synthetic inhibitors of hydroxymethylglutaryl-CoA reductase, an enzyme involved in cholesterol biosynthesis, and the target of important cholesterol-lowering drugs such as lovastatin, simvastatin and fluvastatin (Figure 2) [23,24]. The synthetic inhibitors [25] are structurally very much simpler than these drugs but retain the (4*R*, 6*R*)-β-hydroxy-δ-lactone moiety, which is crucial for bioactivity. For example, compound **4** (Figure 2) has approximately three times the activity of natural compactin [26] and simple analogue **5**, which also shows a small activity, can be envisaged as the tetraketide product of a hypothetical trimodular PKS. This paper demonstrates the feasibility of the stereospecific biosynthesis of compounds **10–13**, containing the target (4*R*, 6*R*)-β-hydroxy-δ-lactone moiety (Figure 1), using designer bimodular PKSs. We also show how this approach can be extended to a trimodular system, generating tetraketides **6** and **7**, which are even closer structural analogues of **5**.

Results and discussion

Assembly of bimodular PKS multienzymes for production of lactones 10 and 11 by recombinant *Saccharopolyspora erythraea*

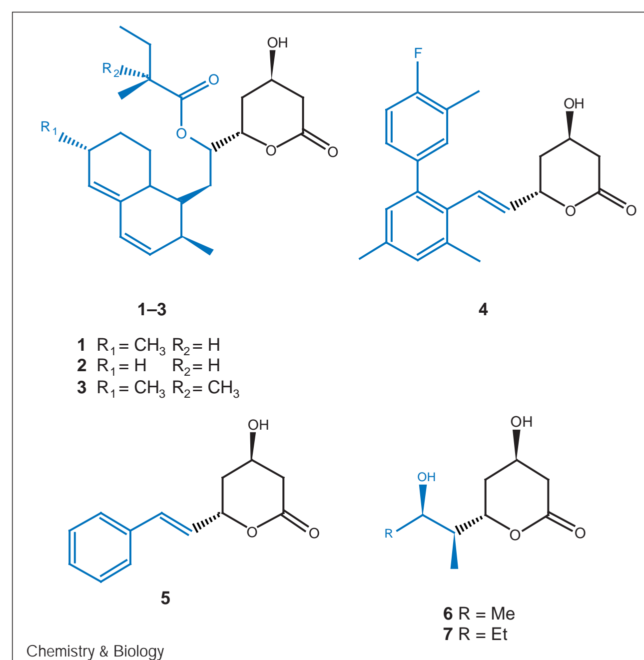
The target compounds **12** and **13** differ from the lactones **8** and **9** normally produced by DEBS1–TE in having no methyl substituent at either C-2 or C-4, and having an opposite configuration of the hydroxyl group at C-3. Several routes were considered for the construction of a suitable hybrid PKS gene starting from DEBS1–TE, all of which exploited the availability of the cloned and sequenced DNA for the entire rapamycin PKS from *Streptomyces hygroscopicus* [5,6]. A number of genetic modifications to module 2 were examined. Substitution of the entire module 2 of DEBS1–TE, from a position in the middle of the ACP1 domain to an equivalent position in

ACP2, with an equivalent region from *rap* module 12, produced the triketide lactone synthase TKS–AR2, which was predicted to give rise to compounds **10** and **11** (Figure 3). To test the possibility that efficient transfer of the growing polyketide chain between modules might be promoted by preserving adjacent ACP and KS domains intact [27], a second hybrid PKS (TKS–AR3) was made, similar to TKS–AR2 except that the acyltransferase (AT), ketoreductase (KR) and ACP domains of *rap* module 12 were fused to the carboxyl terminus of the DEBS KS2 domain, so that the ACP1–KS2 didomain was undisturbed.

To construct TKS–AR2, module 2 was excised from DEBS1–TE to create a diketide synthase in which the DNA for module 1 ACP is fused to that for module 2 ACP at an engineered unique *Bgl*II site, at a position 4–5 amino acids amino-terminal of the 4′-phosphopantetheine attachment site. The entire *rap* module 12 DNA was amplified by the polymerase chain reaction (PCR) and cloned into this *Bgl*II site to create the chimaeric TKS. However, when this gene was expressed in a plasmid expression system in *Streptomyces coelicolor* [14,28] and the broth analysed for the production of triketide lactones using gas chromatography–mass spectrometry (GC–MS), none was found. The reasons for the inactivity of this hybrid synthase could include unfavourable protein–protein interactions between the two halves of the chimaeric ACP1/11. A DEBS1–TE mutant gene was therefore constructed in which the same *Bgl*II site was introduced into the DNA for both ACP1 and ACP2, leading to point mutations Glu1969→Asp and Glu3415→Asp respectively. The gene was expressed in *S. coelicolor* CH999, and it was shown that these two mutations were sufficient to render the synthase inactive.

To construct TKS–AR4, the starting point was a plasmid (pARE62) whose insert comprises the DEBS loading module (AT0-ACP0), linked via an engineered *Nhe*I site in the linker region between the ACP0 and KS1 domains (Figure 4) to the *rap* modules 11 and 12, in turn fused at the carboxyl terminus to the DEBS chain-releasing thioesterase (TE), via a unique *Not*I site in the linker region between *rap* module 12 ACP domain and the TE (Figure 4). The construction of pARE62 is given in the Supplementary material. For TKS–AR3, a unique *Hind*III site was engineered in the linker region between DEBS KS2 and *rap* module 12 AT (Figure 4). The DNA for *rap* module 12 (AT–KR–ACP) and TE was fused to the 3′ end of the DNA for the loading module and module 1 KS2 of DEBS1–TE, in place of the loading module and *rap* module 11–module 12 KS (Figure 3). This hybrid PKS gene, and all the others discussed below, were housed in an SCP2*-based expression plasmid [29] in *S. erythraea* JC2, a strain from which the erythromycin PKS genes have been deleted. This system gives consistently higher

Figure 2



Chemical structures of cholesterol-lowering statins that act as inhibitors of HMG-CoA reductase. lovastatin (**1**), compactin (**2**), simvastatin (**3**). Synthetic and biosynthetic analogues which show statin activity include the aryl analogues **4** and **5**. Compounds **6** and **7** are potential biosynthetic structural analogues of the statin family.

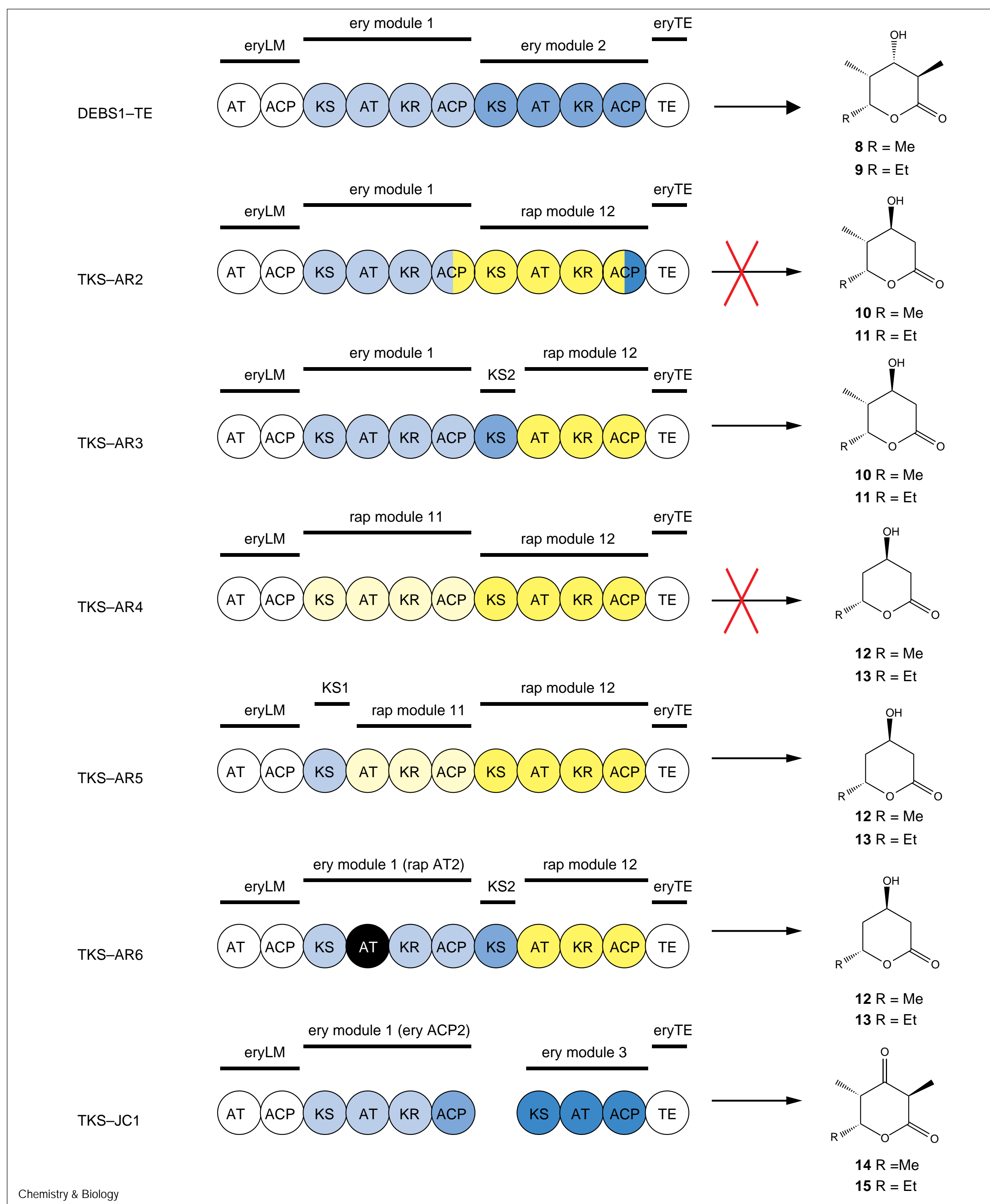
yields of polyketide than are obtained in a comparable expression system [28] in *S. coelicolor*.

The broth from recombinant *S. erythraea* containing this hybrid gene (TKS–AR3) was shown, using GC–MS, to contain two triketide lactones with exactly the masses expected for compounds **10** and **11** (Figure 3). These lactones were purified and their structures were confirmed as **10** and **11**, using nuclear magnetic resonance (NMR), MS and MS–MS analysis. The isolated yields (1.0 mg l^{-1} of **10** and 2.4 mg l^{-1} of **11**) were only about 10% of those expected for the products of DEBS1–TE, but these results clearly established the feasibility of manipulating whole modules to create chimaeric PKS multienzymes. The reasons for the low yields have not yet been investigated in detail, but could include: decreased transcription levels or lower mRNA stability; poor folding or stability of the hybrid multienzyme; or imperfect protein–protein contacts particularly across fusion boundaries.

Assembly of bimodular PKS multienzymes for production of lactones 12 and 13 by recombinant *S. erythraea*

Having established a route to achieve the desired modifications in module 2, several further hybrid enzymes were constructed that were expected to direct the production of the target lactones **12** and **13** in which both branching methyl groups are missing (Figure 3). As

Figure 3



Domain organisation of hybrid bimodular PKS multienzymes made in this study.

Figure 4

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                                          BglII
                                          AGATCT
ACP-01        (ery)     AAAVL GHASAERVPA DQAFAELGVD SLSALELRNR
ACP-06        (ery)     VAAIL GHSSPDAVGQ DQPFTELGFD SLTAVGLRNQ
ACP-11        (rap)     AATVL GHADTSTIPA TTAFKDLGIN SLTAVELRNS
ACP-12        (rap)     AALVL GHADASTIPA AAAFKDLGID SLTAVELRNS

ACP-01:12 (TKS-AR2)     AAAVL GHASAERVPA DQAFADLGIN SLTAVELRNS
ACP-12:06 (TKS-AR2)     AALVL GHADASTIPA AAAFKDLGFD SLTAVGLRNQ

                                              HindIII
                                              AAGCTT
KS2-AT2       (ery)     GEIELADGVR EWSPAADGVR RAGVSAFGVS GTNHVIIAEP
KS12-AT12     (rap)     AGAVQLVTEN QPWPDMGRAR RAGVSSFGIS GTNAHVILES
KS2-AT12 (TKS-AR3)      GEIELADGVR EWSPAADGVR RAGVSSFGIS GTNAHVILES

                                 NotI
                              GCGGCCGC
     ACP   (eryTE)      QELLEFTHSH VAAILGHSSP DAVGQDQPFT ELGFDSLTAV
     ACP   (rap12)      DALLKLVRDS -AALVGHADA STIPAAAAFK DLGIDSLTAV
     ACP (TKS-AR4)      DALLKLVRDS -AAILGHSSP DAVGQDQPFT ELGFDSLTAV

                        NheI
                        GCTAGC
      KS   (ery01)      EPVAVVAMAC RLPGGVSTPE EFWELLSEGR DAVAGLP
      KS   (rap11)      EPLAIVGMAC RLPGGVSSPE DLWRLVESGT DAISDFP
      KS (TKS-AR4)      EPLAIVGMAC RLPGGVSSPE DLWRLVESGT DAISDFP

                                                HindIII
                                                AAGCTT
KS1-AT1       (ery)     SGAISLLDEP EPWPAGARPR RAGVSSFGIS GTNAHAIIEE
KS11-AT11     (rap)     TAGAELVTEN QPWPEIGRPR RAGVSSFGVS GTNAHVILES
KS1-AT11 (TKS-AR5)      SGAISLLDEP EPWPAGARPR RAGVSSFGVS GTNAHVILES

                                              HindIII
                                              AAGCTT
KS2-AT2       (ery)     GEIELADGVR EWSPAADGVR RAGVSAFGVS GTNHVIIAEP
KS11-AT11     (rap)     TAGAELVTEN QPWPEIGRPR RAGVSSFGVS GTNAHVILES
KS2-AT11 (TKS-AR7)      GEIELADGVR EWSPAADGVR RAGVSSFGVS GTNAHVILES
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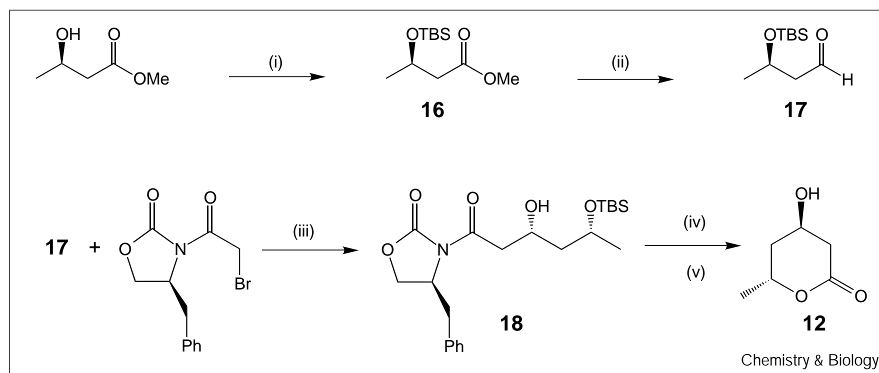
Amino acid sequence around fusion sites in hybrid PKSs based on *ery* and *rap* PKS modules. Restriction enzyme sites were introduced into the encoding DNA at the positions shown. The amino acid sequences of native interdomain linker regions are given for comparison.

already mentioned, plasmid pARE62 contains the hybrid TKS–AR4, in which the entire *rap* modules 11 and 12 are sandwiched between the DEBS loading module and the DEBS chain-terminating TE. These modules are predicted to dictate the formation of a triketide containing acetate extender units with exactly the required stereochemistry at each hydroxyl group [5,6,30]. To provide a direct assessment of the value of preserving adjacent ACP–KS domains intact, a fourth hybrid synthase was constructed (TKS–AR5) in which the *rap* module 11 KS domain was replaced by the DEBS KS1, so that the fusion point in the hybrid lay in the linker region between KS1 and the *rap* module 11 AT domain, at an engineered *Hind*III site (Figure 4). When the gene for multienzyme TKS–AR4 was expressed in recombinant *S. erythraea* JC2, the broths were found, using GC–MS analysis, to contain no trace of triketide lactones. However, low levels of the target molecules **12** and **13** were detected in the broth from the recombinant strain of *S. erythraea* harbouring TKS–AR5. A synthetic sample of the lactone **12** was synthesised in four steps (Figure 5) and was shown to have identical retention time in GC–MS to the material obtained from the broth. Again, it appears that maintaining the juxtaposition of the native ACP0 of the loading module with the KS1 domain was beneficial, although the effect has not yet been precisely quantified. The fusion point in the intermodular loading module-KS1 linker region of AR4 is

Figure 5



Synthesis of (4R,6R)-4-hydroxy-methyltetrahydropyran-2-one [12]. (i) TBS-OTf, Et_3N, DCM, 0°C→rt. (ii) DIBAL-H, DCM, –78°C→rt. (iii) $CrCl_2$, LiI, THF, 0°C→rt. (iv) LiOH, H_2O_2, THF/H_2O, 0°C→rt. (v) 1 M, HCl, THF, 40°C.

very similar to that in a productive fusion of the avermectin PKS loading module and *ery* module 1 [31] and to the fusion of the *ery* module 1 and rifamycin PKS module 5 described recently [32] and correctly predicted to be functional. The fact that the AR4 fusion is inactive may indicate that further work is needed before such predictions can be generally made.

An alternative approach to the target compounds **12** and **13** was pursued. The TKS–AR3 synthase was further modified by replacement of the DEBS AT1 domain, (which is specific for the loading of methylmalonyl-CoA [30]), by the *rap* module 2 AT domain, (which is specific for the loading of malonyl-CoA [14]), to form TKS–AR6 (Figure 3). Oliynyk *et al.* [14] showed that this substitution does not lead to a significant drop in yield of lactone (less than twofold). Plasmid pIB103 contains the DEBS1–TE gene in which a unique *Hind*III site has been introduced in the linker region between the DEBS KS2 and AT2 domains. The DEBS loading module and most of DEBS module 1 were excised on a specific *Nde*I–*Sfu*I fragment and this was replaced with a counterpart fragment from plasmid pMAT2 [14] in which the AT1 domain is replaced by the AT domain from *rap* module 2. Finally, the DEBS module 2 AT, KR and ACP domains, together with an additional 3′ region, were excised on a *Hind*III fragment and replaced by a *Hind*III fragment from plasmid pKS12A (see the Materials and methods section) containing the counterpart domains from *rap* module 12 (Figure 3). Recombinant *S. erythraea* cells containing TKS–AR6 were grown and analysed for the presence of triketide lactones. GC–MS analysis showed the presence of both **12** and **13**, in an approximate ratio of 1:2. The compound identified as **12** showed a retention time and MS fragmentation pattern identical to that of synthetic **12**. The recombinant strain was grown on solid medium and the lactones were extracted from the plates and purified using silica gel chromatography and preparative high-performance liquid chromatography (HPLC). The identity of the purified lactones (about 1 mg l^{-1} of each) was confirmed using MS and NMR spectroscopy as **12** and **13**.

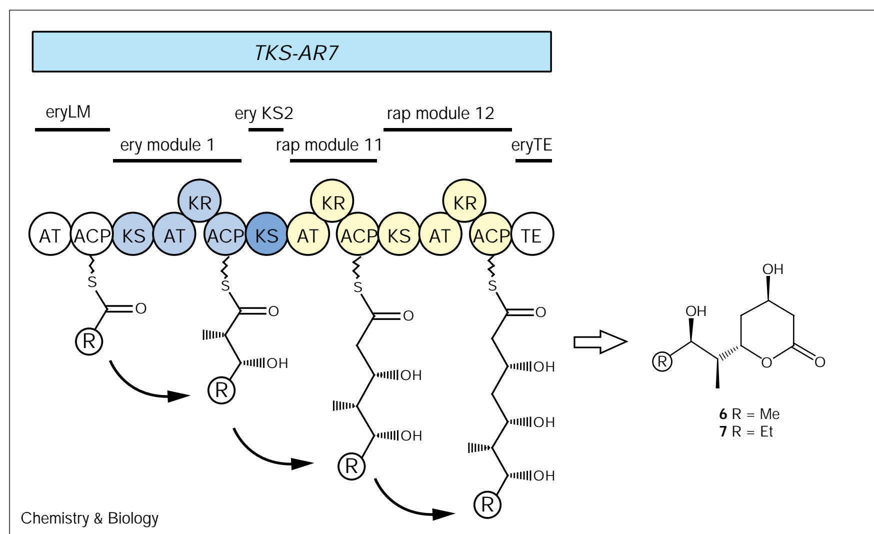
Use of native ACP and KS domains to mediate interprotein polyketide chain transfer

In parallel with the above experiments, we tried another method of preserving a native ACP–KS didomain intact. As indicated in Figure 3, this recombinant PKS, TKS–JC1 contained two proteins derived from DEBS1 and DEBS2. The first consisted of the loading module of DEBS and DEBS module 1 KS, AT, and KR domains, fused to the ACP domain of module 2. The second protein contained DEBS module 3, fused to the chain-terminating TE (see the Materials and methods section for the construction of recombinant plasmids). The recombinant strain was grown on solid medium and the lactones were extracted from the plates and purified using silica gel chromatography and preparative HPLC. The purified lactones were confirmed, by MS and NMR spectroscopy, to be **14** and **15**, both obtained in excellent yields (35 mg l^{-1} of each). This result, in which the productive transfer of a polyketide chain from module 1 to module 3 of DEBS is mediated by the ACP domain of module 2, clearly shows the advantage of keeping the contacts between an ACP and the following KS domain as near native as possible. In a recent paper [32], it has been elegantly shown that, in several cases, the transfer can be effective even if only the region carboxy-terminal of the ACP domain and amino-terminal of the KS domain (the 'linker' regions) are maintained. These results provide interesting confirmation of the predictions embodied in a general model previously proposed [18] for the assembly of modular polyketide synthases, in which the strongest contacts are between two copies of each module, interacting across the subunit interface in a dimer, and the contacts between successive modules are limited to adjacent ACP and KS domains.

Assembly of a trimodular PKS multienzyme for production of lactones 6 and 7 by recombinant *S. erythraea*

A trimodular PKS capable of the synthesis of tetraketides **6** and **7** was then designed (Figure 6) and consisted of *ery* module 1 and *rap* modules 11 and 12, with the *rap* module 11 KS domain replaced by the *ery* KS2 domain. To

Figure 6



Domain organisation of the hybrid trimodular polyketide synthase TKS–AR7.

construct this PKS (TKS–AR7), the amino-terminal region upstream of the AT domain of *rap* module 11 in TKS AR5 (Figure 3) was replaced with the *ery* loading module, *ery* module 1 and domain KS2. The junction covalently linking the modules was constructed in the linker region joining the KS2 to the *rap* module 11 AT at a unique *Hind*III restriction enzyme site as in TKS–AR5 (Figure 4). The final expression plasmid containing the gene for the hybrid trimodular synthase was then integrated into *S. erythraea* JC2. Randomly selected colonies were grown in liquid medium, and the fermentation broth was extracted and analysed for polyketide products. GC–MS analysis showed the presence of only two identifiable polyketides, the target tetraketide lactones **6** and **7**. The recombinant strain of *S. erythraea*/JC2(pMT1) was grown on solid medium and the lactones were extracted from the plates, and purified using silica gel chromatography and preparative HPLC. The identity of the purified lactones was confirmed by MS and NMR spectroscopy as **6** and **7**.

The yields of the products obtained in the above experiments were not optimised, and varied from excellent (35 mg l^{-1} of purified triketide for each of **14** and **15**) to modest (about 1 mg l^{-1} of each tetraketide **6** and **7** to barely detectable (in the case of triketides **10** and **11** produced by TKS–AR4). Although the emerging rules for constructing hybrid type I modular PKS multienzymes are engagingly simple, some further work is needed to define the optimum fusion sites to choose within the linker regions between domains (see also the recent elegant work of Gokhale *et al.* [32]); to identify the most versatile natural modules and domains to deploy; and to enhance the production and stability of recombinant PKS proteins. Thus, the relatively high yield we have found for the production of **14** and **15** by TKS–JC1 focuses attention on the linker region between the reductive domains (dehydratase–enoylreductase–ketoreductase) and the ACP domain, in future efforts to assemble modules systematically. Part of the superior efficiency of TKS–JC1 may arise from the fact that transfer occurs between modules housed in different multienzymes, but further work is needed to clarify this issue. DNA shuffling methodologies can also be employed if a suitable assay is available [33,34].

The successful specific production of triketides and tetraketides using rationally designed modular PKSs is obviously encouraging for further work aimed at producing **5** in a single-step fermentation. This future work needs to address two specific problems: a synthase is required in which the first module employs an acetate chain extender and processes this as far as a double bond rather than a hydroxyl function. These changes could be achieved by rational engineering of *ery* module 1 or by using another appropriate module. Futhermore, a loading module is required that accepts CoA esters of benzoic and other aryl acids; and finally, a suitable source of aryl acid units must be present, either by biosynthesis or by addition to the fermentation, together with a mechanism for their activation.

Significance

Modular polyketide synthases (PKSs) are potentially valuable for the biosynthesis of novel complex polyketides. The demonstration that a designed hybrid trimodular polyketide synthase catalyses the predicted formation of a simple analogue of the cholesterol-lowering statins illustrates the principle of designing a PKS to conduct

chiral synthesis. This artificial synthase is multimodular and is therefore more suitable for designed alterations of structure than the single modular PKS implicated in the natural biosynthesis of lovastatin [35]. The ability to manipulate whole modules is of particular importance for the *de novo* assembly of complex molecules. The results show that the interaction between an acyl carrier protein (ACP) domain and the adjacent ketosynthase (KS) domain is crucial for efficient intermodule chain transfer, both between modules housed in the same multienzyme and between modules in successive multienzymes of the PKS. It is not yet apparent, however, whether the entire ACP–KS didomain region has to be preserved. The hybrid PKSs are significantly less efficient than the natural PKS multienzymes from which they have been derived, but there are obvious opportunities for further optimisation, especially through the use of alternative domains and modules from natural sources and the given choice of alternative fusion sites in the inter-domain linker regions.

Materials and methods

Strains and plasmids

S. erythraea/JC2 was maintained as described previously [29]. Routine cloning and transformation procedures were as previously described for *Escherichia coli* [36] and for *Streptomyces* [37,38]. Electrocompetent cells of *E. coli* DH10B strain were made as described previously [39]. X-gal was obtained from Novabiochem. Agarose (electrophoresis grade) was obtained from Life Technologies Inc., casamino acids and tryptone were from Difco laboratories. Glucidex (MD30E), soya flour and beet molasses for SM3 culture media were the kind gifts of Glaxo Wellcome, UK. All antibiotics were bought from Sigma Chemical Company, MO, USA.

DNA manipulations

PCR reactions were performed on a programmable Robo Cycler Gradient 40 (Stratagene, USA) according to a modification of a protocol described earlier [40]. Automated DNA sequencing was carried out on double-stranded DNA templates using an automated ABI 373A sequencer (Applied Biosystems). RecA protein was purchased from Promega.

Chemical analysis

^{1}H NMR spectra were recorded at 600 MHz on a Bruker DRX-600, at 500 MHz on a Bruker DRX-500, at 400 MHz on a Bruker AM-400 or Bruker DRX-400, and at 250 MHz on a Bruker AC-250. ^{13}C NMR spectra were recorded at 100 MHz on a Bruker AM-400 or at 100 MHz on a Bruker DRX-400. ^{1}H NMR and ^{13}C NMR spectra were referenced internally to $CHCl_3$ (7.27 and 77.5 ppm respectively). *J* Values are given in Hz. Chromatography was carried out using Merck Kieselgel 60 (40–63 μm). GC–MS was performed on a Finnigan MAT GCQ instrument. Analytical and preparative reverse-phase HPLC–MS analysis was carried out using Phenomenex Prodigy 5 μ ODS3 100 Å columns with the following dimentions: 250×4.6 mm (analytical), 250×10.0 mm (semi-preparative) and 250×21.2 mm (preparative) on a Finnigan MAT LCQ instrument. Mass spectra were recorded on a Kratos MS 890 double focussing magnetic sector MS (EI) and a Bruker Bio Apex II Fourier Transform Ion Cyclotron Resonance (FT-ICR, 4.7 Tesla), (ESI). Optical rotations were recorded on a Perkin Elmer 241 polarimeter. Solvents were dried under standard conditions.

Growth medium

Special Medium 3 (SM3) (for 1 l); Glucose 5 g, MD30E-glucidex 50 g, soya bean flour 25 g, beet molasses 3 g, K_2HPO_4 0.25 g, $CaCO_3$ 2.5 g, Milli-Q water to 1 l and pH adjusted to 7.0 with KOH.

Construction of expression plasmids pBgAR4 and pARE62 for the hybrid synthases TKS–AR2 and TKS–AR4 respectively

The construction of these expression plasmids is described in detail in the Supplementary material.

Construction of plasmid pO15-111224 for the hybrid synthase TKS-AR5

Plasmid pKS11A (the construction of which is described in the supplementary material) was cut with *Hind*III and the 10 kbp fragment containing the gene encoding for *rap* module 11, *rap* module 12 and *ery* TE was ligated to the vector pIBO15 [27] previously cut with *Hind*III. *E. coli* DH10B cells were transformed with the ligation mixture and selected colonies were screened for their plasmid content. The desired plasmid pO15-111224 was identified by restriction mapping.

Construction of plasmid pIBM1

Plasmid pMAT2 [14] was cut with *Nde*I and *Sfu*I and the 4.6 kbp fragment containing the gene encoding the *ery* loading module, and *ery* module 1 with the *rap* AT2 domain, was ligated to plasmid pIB103 previously cut with *Nde*I and *Sfu*I. Plasmid pIB103 contains the gene for the DEBS1-TE synthase. The ligation mixture was used to transform *E. coli* DH10B cells and selected colonies were screened for their plasmid content. The desired plasmid pIBM1 was identified by restriction mapping.

Construction of plasmid pAT-1224 for the hybrid synthase TKS–AR6

Plasmid pKS12A (the construction of which is described in the Supplementary material) was cut with *Hind*III and the 5.4 kbp fragment containing the gene encoding *rap* module 12 and *ery* TE was ligated to plasmid pIBM1 previously cut with *Hind*III. *E. coli* DH10B cells were transformed with the ligation mixture and selected colonies were screened for their plasmid content. The desired plasmid pAT-1224 was identified by restriction mapping.

Construction of plasmid p103-1224 for the hybrid synthase TKS–AR3

Plasmid pKS12A was digested with *Hind*III and the 5.4 kbp fragment containing the gene encoding *rap* module 12 and *ery* TE was ligated to plasmid pIB103 previously cut with *Hind*III. *E. coli* DH10B cells were transformed with the ligation mixture and selected colonies were screened for their plasmid content. The desired plasmid p103-1224 was identified by restriction mapping.

Construction of plasmid p103-111224 for the hybrid synthase TKS-AR7

Plasmid pKS11A was cut with *Hind*III and the 10.0 kbp fragment containing the fragment encoding *rap* module 11 (minus the KS11 domain), *rap* module 12 and *ery* TE was ligated to plasmid pIB103 previously cut with *Hind*III. *E. coli* DH10B cells were transformed with the ligation mixture and colonies were selected for resistance to ampicillin. Plasmid DNA isolated from ampicillin-resistant clones was mapped by restriction enzyme digestion and the desired plasmid was designated p103-111224.

Construction of pE1

A PCR product containing DNA encoding for the erythromycin KR1 domain was obtained using oligonucleotides 5′–GCCTAGGCACCG-GAGCACGCCGGGTGCCCTT-3′ and 5′–CGTTAACGCGCCCAC-CCGCGGTTCGGCCGGCGCCT-3′ using pNTEP2 [14] as template. This product was cloned into pUC18 previously digested with *Sma*I to produce plasmid pCRE1. This plasmid was digested with *Avr*II and *Hpa*I and the 1.8 kbp insert was cloned into pJLK14 (construction of this plasmid will be described elsewhere) previously digested with *Avr*II and *Hpa*I to obtain plasmid pE1. Plasmid pE1 encodes a triketide synthase D1TE where KR2 has been replaced by KR1.

Construction of pDKS

Plasmid pEI was digested with *Nsp*V and religated, the resulting plasmid encodes a diketide synthase containing *ery* LM, KS1, AT1, KR1 and ACP-TE from D1TE.

Construction of pDKSH for the hybrid synthase TKS-JC1
A PCR product containing DNA encoding from the start of the erythromycin ACP domain of module 2 to the end of the inactive KR domain of module 3 was obtained using oligonucleotides 5′-ATGTTAACGGGTCTGCCGCGTGCCGAGCGGAC-3′ and 5′-ATGTTAACCGTTGTGCCGGCTCGCCGGTCGGTCC-3′ using plasmid pBK25 [3] as template. This product was cloned into pUC18 previously digested with *Sma*I to produce plasmid pMOD3. This plasmid was digested with *Hpa*I and the 6.1 kbp insert was cloned into pDKS previously digested with *Hpa*I to obtain plasmid pDKSH. This plasmid encodes a two protein triketide synthase containing, in one polypeptide ery LM, KS1, AT1, KR1 and ACP2 and in the second polypeptide, KS3, AT3, the inactive KR3 domain and ACP-TE from D1TE.

Construction of S. erythraea*/JC2(pO15-111224),* S. erythraea*/JC2(pAT-1224),* S. erythraea*/JC2(p103-1224),* S. erythraea*/JC2 (pMT1) and* S. erythraea*/JC2(pDKSH)*
S. erythraea/JC2 protoplasts were transformed with the expression plasmids and colonies were selected through resistance to thiostrepton upon integration of the plasmid into the *S. erythraea* chromosome. Single transformants were picked and grown on 'tap-water medium' plates supplemented with thiostrepton following which single transformants were grown in SM3 liquid/solid medium supplemented with 5 µg ml^{-1} of thiostrepton as described in the analysis.

Analysis
*Isolation of (4R,5R,6R) 5,6-dimethyl-4-hydroxy-tetrahydropyran-2-one (**10**) and (4R,5R,6R) 6-ethyl-4-hydroxy-5-methyl-tetrahydropyran-2-one (**11**).* *S. erythraea*/JC2 (p103-1224) was grown on five special medium (SM3) agar plates, from an initial inoculum in tryptic soy broth (TSB) liquid medium (30°C, 300 rpm, 3 days). After 24 days the agar was extracted with ethyl acetate/2% formic acid (5 × 250 ml). The combined organic extracts were neutralised with $NaHCO_3$, dried over Na_2SO_4 and concentrated *in vacuo*. The concentrated extract was passed through silica gel eluting with diethyl ether (300 ml) followed by ethyl acetate (300 ml). The eluent was concentrated and purified by silica column chromatography (EtOAc/Petroleum ether, 2:1) Appropriate fractions were combined and further purified by preparative reverse phase HPLC–MS (H_2O, MeOH) to yield lactones **10** and **11** (1.0 mg l^{-1} and 2.4 mg l^{-1} respectively).

*(4R,5R,6R) 5,6-Dimethyl-4-hydroxy-tetrahydropyran-2-one (**10**).* ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.90 (dq, 1 H, J = 3.0 and 6.8, 6–H), 4.06 (m, 1 H, 4–H), 2.82 (dd, 1 H, J = 5.5 and 18.3, 3–H_{ax}), 2.53 (ddd, 1 H, J = 0.9, 3.4 and 18.3, 3–H_{eq}), 1.91 (ddq, 1 H, J = 3.0 and 7.3, 5–H_{eq}), 1.33 (d, 3 H, J = 6.8, C6-Me), 0.97 (d, 3 H, J = 7.3, C5-Me); MS (ESI) m/z $C_7H_{12}O_3Na$ calc'd 167.0675080, found 167.0678634 [M + Na]$^+$.

*(4R,5R,6R) 6-Ethyl-4-hydroxy-5-methyl-tetrahydropyran-2-one (**11**).* ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.61 (ddd, 1 H, J = 3.0, 5.5 and 8.3, 6–H), 4.06 (m, 1 H, 4–H), 2.82 (dd, 1 H, J = 5.5 and 18.3, 3–H), 2.54 (dd, 1 H, J = 3.4 and 18.3, 3–H), 1.96 (m, 1 H, 5–H_{eq}), 1.78 (ddq, 1 H, J = 7.3, 8.3 and 13.6, C*H*HCH$_3$), 1.55 (ddq, 1 H, J = 5.5, 7.3 and 13.6, CH*H* CH$_3$), 1.03 (t, 3 H, J = 7.3, CH_2CH_3), 0.94 (d, 3 H, J = 7.3, C5–Me). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 170.3 (2–C), 79.5 (6–C), 68.8 (4–C), 37.1, 35.6, 24.9 (*C*H$_2$CH$_3$), 10.2 (CH_3), 10.0 (CH_3). (ESI) m/z $C_8H_{14}O_3Na$ calc'd 181.0831880, found 181.0835134 [M + Na]$^+$.

Isolation of (4R,6R) 4-hydroxy-6-methyl-tetrahydropyran-2-one (12) and (4R,6R) 6-ethyl-4-hydroxy-tetrahydropyran-2-one (13). *S. erythraea*/JC2(pAT-1224) was grown on six SM3 agar plates, from an initial inoculum in TSB liquid medium (30°C, 300 rpm, 3 days). After 29 days the agar was extracted with ethyl acetate/2% formic acid (6 × 250 ml). The combined organic extracts were neutralised with $NaHCO_3$, dried over Na_2SO_4 and concentrated *in vacuo*. The concentrated extract was passed through silica gel eluting with diethyl ether (1.5 l), ethyl acetate (2 l) and the eluent was concentrated. Reverse-phase HPLC–MS (H_2O, MeOH) afforded lactones **12** and **13**. Fractions containing required products were extracted into DCM. The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo to yield lactones **12** and **13** (about 1 mg l^{-1} of each). The GC–MS trace of a crude broth extract containing lactone **12** was recorded. The peak corresponding to **12** had a retention time identical to that of the synthetic standard triketide **12**. A sample of the crude extract was 'spiked' (3:1 v/v) with a sample of its synthetic standard. The peak corresponding to **12** increased in intensity with respect to all other peaks in the extract (as confirmed by peak integration). This allowed for verification of the identity of the acetate-starter lactone (**12**).

(4R,6R) 4-Hydroxy-6-methyl-tetrahydropyran-2-one (12). ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.84 (ddq, 1 H, J = 3.0, 6.4 and 11.1, 6–H), 4.39 (m, 1 H, 4–H), 2.74 (dd, 1 H, J = 5.1 and 17.5, 3–H_{ax}), 2.61 (ddd, 1 H, J = 1.7, 3.8 and 17.5, 3–H_{eq}), 1.97 (dddd, 1 H, J = 1.7, 3.0, 3.8 and 14.5, 5–H_{eq}), 1.75 (ddd, 1 H, J = 3.4, 11.1 and 14.5, 5–H_{ax}), 1.41 (d, 3 H, J = 6.4, C6–Me); MS (ESI) m/z $C_6H_{10}O_3Na$ calc'd 153.0522134, found 153.0520930 [M + Na]$^+$.

*(4R,6R) 6-Ethyl-4-hydroxy-tetrahydropyran-2-one (**13**).* ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.67 (dddd, 1 H, J = 3.0, 5.3, 7.1, and 11.3, 6–H), 4.40 (m, 1 H, 4–H), 2.75 (dd, 1 H, J = 5.1 and 17.5, 3–H_{ax}), 2.62 (ddd, 1 H, J = 1.7, 3.8 and 17.5, 3–H_{eq}), 1.96 (dddd, 1 H, J = 1.7, 3.0, 3.4 and 14.5, 5–H_{eq}), 1.75 (m, 2 H, 6–C*H*H, 5–H_{ax}), 1.67 (m, 1 H, C6–C*H*H), 1.03 (t, 3 H, J = 7.3, CH$_2$C*H*$_3$); (ESI) m/z $C_7H_{12}O_3Na$ calc'd 167.0678634, found 167.0681550 [M + Na]$^+$.

*Isolation of (4R,6R) 4-hydroxy-6-(1′S-methyl-2′R-hydroxy)propyl-tetrahydro-pyran-2-one (**6**) and (4R,6R) 6-(1′S-methyl-2′R-hydroxy)butyl-4-hydroxy-tetrahydro-pyran-2-one (**7**).* *S. erythraea*/JC2 (pMT1) was grown on five SM3 agar plates, from an initial inoculum in TSB liquid medium (30°C, 300 rpm, 3 days). After 25 days the agar was extracted with ethyl acetate/2% formic acid (5 × 250 ml). The combined organic extracts were neutralised with $NaHCO_3$, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography eluting with diethyl ether (300 ml), DCM (300 ml) and DCM/methanol (8:2 × 1 l, 7:3 × 1 l and 6:4 × 1 l). The concentrated eluent was further purified by reverse-phase HPLC–MS (H_2O, MeOH), and concentration of appropriate fractions *in vacuo* yielded lactones **6** and **7** (about 1 mg l^{-1} of each).

*(4R,6R) 4-Hydroxy-6-(1′S-methyl-2′R-hydroxy)propyl-tetrahydro-pyran-2-one (**6**).* ^{1}H NMR (600 MHz, $CDCl_3$) δ = 4.89 (dt, 1 H, J = 3.8 and 10.9, 6–H), 4.44 (m, 1 H, 4–H), 4.08 (dq, J = 3.3 and 6.2, 2′–H), 2.74 (dd, 1 H, J = 4.8 and 17.6, 3–H_{ax}), 2.63 (ddd, 1 H, J = 1.9, 3.3 and 17.6, 3–H_{eq}), 1.96 (m, 2 H, 2 × 5–H), 1.65 (m, 1 H, 1′–H) 1.25 (d, 3 H, J = 6.2, 3′–Me), 1.04 (t, 3 H, J = 7.3, C1′–Me); MS (ESI) m/z $C_9H_{16}O_4Na$ calc'd 211.0933010, found 211.0940774 [M + Na]$^+$.

*(4R,6R) 6-(1′S-Methyl-2′R-hydroxy)butyl-4-hydroxy-tetrahydro-pyran-2-one (**7**).* ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.85 (ddd, 1 H, J = 4.7, 6.8 and 8.1, 6–H), 4.43 (m, 1 H, 4–H), 3.75 (ddd, 1 H, J = 3.0, 5.1 and 7.7, 2′–H) 2.74 (dd, 1 H, J = 5.1 and 17.5, 3–H), 2.65 (m, 1 H, 3–H), 1.95 (m, 2 H, 2 × 5–H), 1.75 (ddq, 1 H, J = 3.0, 4.7 and 7.3, 1′–H), 1.55 (m, 2 H, 2 × 3′–H), 1.02 (d, 3 H, J = 7.3, C1′–Me), 0.96 (t, 3 H, J = 7.3, 4′–Me); MS (ESI) m/z $C_{10}H_{18}O_4Na$ calc'd 225.1095180, found 225.1097274 [M + Na]$^+$.

Synthesis of (3R)-3-(t-Butyldimethylsiloxy)-butyrate (16) [41]. Methyl (3*R*)-3-hydroxybutyrate (3.8 ml, 34 mmol, 1.0 eq.) was dissolved in DCM (50 ml), cooled to 0°C and stirred under argon. Following the addition of *t*-butyldimethylsilyltrifluoromethylsulfonate (TBS-triflate, 12 ml, 51 mmol, 1.5 eq.), the mixture was stirred for 10 min and triethylamine (12 ml, 85 mmol, 2.5 eq.) was added. The solution was allowed to warm to room temperature, stirred for 2.5 h and quenched with brine (50 ml). The aqueous layer was extracted with DCM (3 × 50 ml). Combined organic extracts were washed with NaOH (pH 11) and dried over Na_2SO_4. Flash column chromatography (R_f 0.53, 5% ethyl acetate in light petroleum) afforded the pure product **16** as a colourless oil

(5.0 g, 64%); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.27 (m, 1 H, 3–H), 3.66 (s, 3 H, OMe), 2.48 (dd, 1 H, J = 7.7 and 14.5, 2-C*H*H), 2.37 (dd, 1 H, *J* = 5.3 and 14.5, 2–CH*H*), 1.19 (d, 3 H, *J* = 6.1, 4–Me), 0.86 (m, 9 H, *t*-Bu), 0.06 (s, 3 H, Si–CH_3), 0.04 (s, 3 H, Si–CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 172.1, 65.8, 51.4, 44.7, 25.7, 23.9, 17.9, –4.5, –5.1; υ_{max} (thin film)/cm^{-1} 1742.6 (C = O); $[\alpha]_D$ –28.2 (c 0.44 $CHCl_3$); MS (ESI) *m/z* calc'd 233.15772, found 233.15772 [M]$^+$.

Synthesis of (3R)-3(-t-Butyldimethylsiloxy)-butanal (17) [41]. (3*R*)-3-(*t*-Butyldimethylsiloxy)-butyrate (**16**) (5.0 g, 22 mmol, 1.0 eq) was dissolved in DCM (100 ml) and cooled to (78°C under argon. DIBAL-H (22 ml, 22 mmol, 1.0 eq of a 1 M solution in DCM) was added and the solution was stirred at –78°C for a further 1 h. The reaction was quenched by the addition of a saturated solution of Rochelle's salt (potassium sodium tartrate tetrahydrate, 50 ml), allowed to warm to room temperature, and stirred for 1 h. The aqueous layer was extracted with DCM (3 × 100 ml). The combined organic extracts were dried over Na_2SO_4 and reduced *in vacuo*. Flash column chromatography (2% ethyl acetate in light petroleum) afforded the pure product **17** as a colourless oil (3.6 g, 82%); R_f 0.47, 5 % ethyl acetate in light petroleum; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.80 (t, 1 H, *J* = 2.4, 1–H), 4.35 (m, 1 H, 3–H), 2.55 (ddd, 1 H, *J* = 2.8, 6.8, and 15.7, 2–C*H*H), 2.46 (ddd, 1 H, *J* = 2.0, 5.0, and 15.7, 2–CH*H*), 1.24 (d, 3 H, *J* = 6.2, 4–Me), 0.87 (s, 9 H, *t*-Bu), 0.07 (s, 3 H, Si–CH_3), 0.06 (s, 3 H, Si–CH_3); ^{13}C (100 MHz, $CDCl_3$) δ = 202.2, 64.5, 53.0, 25.7, 24.2, 17.9, –4.4, –5.0; υ_{max} (thin film)/cm^{-1} 1729.2 (C = O) $[\alpha]_D$ –10.8 (c 5.15 $CHCl_3$); MS (ESI) *m/z* calc'd 225.1281274, found 225.1286420 [M + Na]$^+$.

Synthesis of (4S)-3-[(3′R,5′R)-5′-[(t-Butyldimethylsilyl)oxy]-3′-hydroxyhexanoyl]-4-phenylmethyl-2-oxazolidinone (18) [42]. (4*S*)-3-Bromoacetyl-4-phenylmethyl-2-oxazolidinone [41] (1.2 g, 4.0 mmol, 1.0 eq.) was dissolved in THF (15 ml) and stirred at 0°C. (3*R*)-3(-*t*-Butyldimethylsiloxy)-butanal (**17**) (0.90 g, 4.4 mmol, 1.1 eq.) was dried azeotropically over benzene, dissolved in THF (5 ml) and added to the mixture. Anhydrous chromium(II) chloride (1.24 g, 10.1 ml, 1.0 eq.) and lithium hydroxide (0.05 g, 0.4 mmol, 0.1 eq.) were added and the reaction mixture was allowed to warm to room temperature. The reaction was stirred under argon for 3 h and quenched by the addition of brine (15 ml). The aqueous layer was extracted with diethyl ether (3 × 15 ml). The combined organic extracts were washed with distilled water, dried over Na_2SO_4, filtered through celite and concentrated *in vacuo*. The crude products were purified using flash column chromatography (5% ethyl acetate in light petroleum affording 115 mg of the side-product (4*S*)-3-acetyl-4-phenylmethyl-2-oxazolidinone. Further purification of 190 mg of crude material by HPLC yielded 57 mg of the major diastereomer (**18**);

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.29 (m, 5 H, Ph), 4.68 (m, 1 H, 4–H), 4.29 (m, 1 H, 3′–H), 4.15 (m, 3 H, 2 × 5–H, 5′–H), 3.30 (dd, 1 H, *J* = 3.4 and 13.5, 4–H_A), 3.12 (m, 2 H, 2 × 2′–H), 2.79 (dd, 1 H, *J* = 9.5 and 13.5, 4–H_B), 1.77 (m, 1 H, 4′–H_A), 1.62 (m, 1 H, 4′–H_B), 1.20 (d, 3 H, *J* = 6.1, 6′–Me), 0.89 (9 H, s, *t*-Bu), 0.10 (6 H, s, Si(CH_3)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 172.1, 153.4, 135.2, 129.5, 129.0, 127.4, 68.2, 66.9, 66.3, 55.1, 45.7, 43.0, 37.8, 25.9, 24.1, 18.0, –4.4, –4.0; υ_{max} (thin film)/cm^{-1} 3500 (OH), 1784 (1–C = O), 1694 (1′–C = O) cm^{-1}; $[\alpha]_D$ –12.5 (c 0.78 $CHCl_3$); MS (ESI) *m/z* calc'd 444.2176684, found 444.2174280 [M + Na]$^+$.

Synthesis of (4R,6R)-4-hydroxy-methyltetrahydropyran-2-one (12) [43]. (4*S*)-3-[(3′*R*,5′*R*)-5′-[(*t*-Butyldimethylsilyl)oxy]-3′-hydroxyhexanoyl]-4-phenylmethyl-2-oxazolidinone (**18**) (0.02 g, 0.04 mmol, 1 eq.) was dissolved in THF/H_2O (4 ml, 3:1) and stirred under argon at 0°C and hydrogen peroxide (20 µl, 0.19 mmol, 4.5 eq.) and lithium hydroxide (2.3 mg, 0.01 mmol, 2.3 eq.) were added. The solution was warmed to room temperature and stirred for 1.5 h. The reaction was quenched by addition of sodium sulphite solution (170 µl, 0.26 mmol, 6 eq.), acidified (pH 1) by the dropwise addition of 1 M HCl and stirred for 2 h at 40°C. Following the addition of saturated brine (15 ml) the mixture was extracted with DCM (10 × 50 ml), dried over Na_2SO_4 and concentrated *in vacuo*. Flash column chromatography (light petroleum, light petroleum:ethyl acetate, 7:3) afforded the lactone **12** (1.0 mg, 18%) as a colourless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.85 (ddq, 1 H, *J* = 3.0, 6.4 and 11.1, 6–H), 4.40 (m, 1 H, 4–H), 2.74 (dd, 1 H, *J* = 5.1 and 17.9, 3–H_{ax}), 2.61 (ddd, 1 H, *J* = 1.7, 3.8 and 17.9, 3–H_{eq}), 1.97 (dddd, 1 H, *J* = 1.7, 3.0, 5.5 and 15.5, 5–H_{eq}), 1.75 (ddd, 1 H, *J* = 3.4, 11.1 and 14.5, 5–H_{ax}), 1.40 (t, 3 H, *J* = 6.4, C6–Me); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 170.2, 72.1, 62.9, 38.4, 37.7, 21.3; υ_{max} (thin film)/cm^{-1} 1725.0 (C = O) cm^{-1}; $[\alpha]_D$ +21 (c 0.05 $CHCl_3$; Lit.[44] $[\alpha]_D$ +23); MS (ESI) *m/z* calc'd 153.0522134, found 153.0523200 [M + Na]$^+$.

Supplementary material
Supplementary material including details about the construction of expression plasmids pBgAR4 and pARE62 for the hybrid polyketide synthases TKS–AR2 and TKS–AR4, respectively, is available at http://current-biology.com/supmat/supmatin.htm.

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