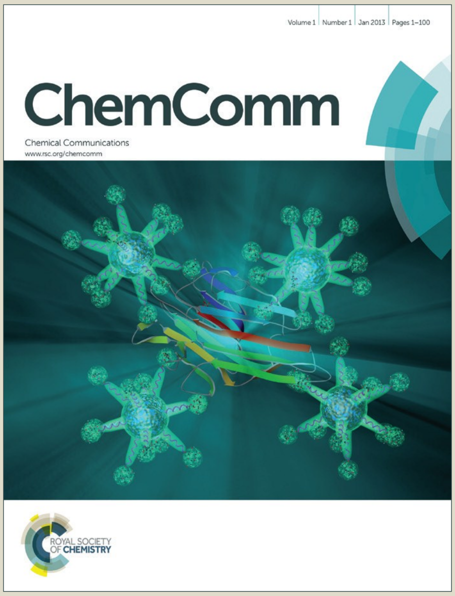
Volume 1 | Number 1 | Jan 2013 | Pages 1–100
ChemComm
Chemical Communications
www.rsc.org/chemcomm
ROYAL SOCIETY OF CHEMISTRY

ChemComm

COMMUNICATION

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Broadening substrate specificity of a chain-extending ketosynthase through a single active-site mutation

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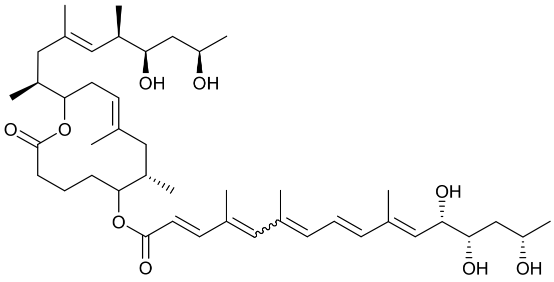
An *in vitro* model system based on a ketosynthase domain of the erythromycin polyketide synthase was used to probe the apparent substrate tolerance of ketosynthase domains of the mycolactone polyketide synthase. A specific residue change was identified that led to an emphatic increase in turnover of a range of substrates.

The manipulation of biosynthetic pathways provides a useful source of novel analogues of pharmaceutically-important, complex natural products,[1] as well as cost-effective and sustainable routes to known compounds.[2,3,4] Modular type I polyketide biosynthesis in particular provides an attractive platform for producing rationally engineered biocatalysts that can generate organic molecules of specified shape and size.[5]

In the biosynthesis of complex reduced polyketides, such as mycolactone (**1**) and erythromycin A (**2**) (Fig. 1), by actinomycete bacteria, carbon chains of specific length are produced from small monomers on assembly-line modular polyketide synthase (PKS) multienzymes, each successive module catalysing a different cycle of chain extension.[6,7] The key step of carbon-carbon bond formation involves a thioester-templated Claisen condensation reaction, catalyzed by a ketosynthase (KS), between the growing chain tethered to the KS active site, and a chain-extending monomer borne on an acyl carrier protein (ACP) domain. Selection and loading of the chain-extending monomer is carried out by an acyltransferase (AT) domain. The β-ketoacyl-ACP intermediate resulting from condensation may undergo reduction before transfer to the next module, the degree of reduction depending on the presence of ketoreductase, dehydratase and enoyl reductase enzymes in each respective module. Many examples of domain or module replacement, insertion and deletion to give functional chimæric PKSs have been described.[8] Unfortunately these hybrid modular PKSs are often much less efficient than the parent native PKS. Early model studies showed that KSs have some intrinsic tolerance for different substrates.[9,10] However, the inherent substrate specificity of the KS in the adjacent downstream module may often limit the activity of hybrid modular PKS systems.[11-13] The role of KS specificity in assembly-line polyketide biosynthesis has been previously explored in *trans*-AT PKSs, which lack in-built AT domains in each module.



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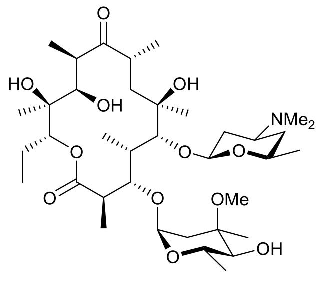

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Fig. 1. Chemical structures of mycolactone and erythromycin.

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Bio-informatic analysis of their KS sequences has shown that they form distinct clades corresponding to the chemistry of their substrates.[14] This structure-selectivity correlation, which has been confirmed by *in vitro* functional analysis,[15-18] implies that native *trans*-AT KS domains may be generally poor catalysts for extension of non-natural chains. In contrast, the sequences of KS domains from a given *cis*-AT PKS tend to form a single clade, irrespective of the chemical nature of the substrate at each stage of elongation.[14] Although apparently more promising as catalysts operating within hybrid PKS assemblies, the determinants of KS active site specificity in *cis*-AT systems remain rather poorly understood.[8] A better understanding of individual structural features that determine specificity would greatly assist the re-engineering of chimæric systems to improve function.

A valuable framework for detailed analysis of KS specificity has been provided by X-ray crystal structures determined for several KS domains.[16,19-22] Structure-based sequence alignment of both *cis*-AT and *trans*-AT KS domains has revealed three variable regions within KS domains; a "clasping loop", a "dimer interface loop" and an "active site cap" (Fig. S1).[21] In an earlier comparison of the erythromycin PKS (6-deoxyerythronolide B synthase, DEBS) KS5AT5 and KS3AT3 structures,[20] the difference in conformation adopted by the dimer interface loop was similarly suggested to contribute to the different substrate specificity of KS3 and KS5. Roles in determining substrate specificity in *trans*-AT KS domains have been plausibly assigned to the amino acid residue immediately N-terminal of the essential active site Cys.[15,17,18] but there have been no equivalent successes for the KS domains of *cis*-AT PKSs. Also, at the outset of our work there was no evidence that engineered sequence changes in *cis*-AT KSs might improve tolerance for non-natural acyl-ACP substrates.

A new approach to this question is suggested by the existence of a natural *cis*-AT PKS showing an exceptionally high level of sequence identity (>98%) between KS domains in different extension modules, even though the KSs act on substrates that vary significantly in chain length and chemical functionality. This is the PKS responsible for generating mycolactone (**1**) in *Mycobacterium ulcerans*, the causative agent of Buruli ulcer.[23-25] This multienzyme appears to offer a unique natural example of broad KS substrate tolerance. In principle, mycolactone modules might serve as universal building blocks in combinatorial polyketide biosynthesis. Unfortunately, the slow-growing mycolactone producer is intractable for genetic manipulation,[26] and the PKS is not active upon heterologous expression.[27] Insertion of a *myc* KS domain into a heterologous PKS assembly line to replace the resident KS is not expected to be effective, due to deleterious effects on KS:AT and KS:ACP protein:protein interfaces.

To learn from the remarkable tolerance of *myc* KS domains, we instead analysed *in silico* the active site differences between modelled mycolactone KS domains and the experimentally-determined crystal structure of the DEBS KS3AT3 didomain (EryKS3AT3).[20] Guided by this comparison, we then replaced specific amino acids in the EryKS3 active site by their mycolactone KS counterparts, and determined the condensation activity of each mutant KS3 enzyme towards a panel of surrogate thioester substrates *in vitro*. We report here that although most of the mutants conserved the catalytic properties of the parent enzyme, the replacement of a specific alanine residue by tryptophan markedly improved both catalytic turnover and the ability of the enzyme to act on non-natural substrates, encouraging the view that the performance of chimæric *cis*-AT PKS multienzymes may be improved by active-site engineering.

Results and discussion

In silico comparison between the KS domains of the mycolactone PKS and the EryKS3AT3 crystal structure

An *in silico* model of the KS domain from MlsA2 was generated using Phyre2[29] and compared with the EryKS3AT3 didomain crystal structure,[20] to identify amino acid residues likely to define the extended substrate binding pocket of the KS domain. The catalytically essential residues Cys202, His337 and His377 (EryKS3 domain numbering) occupy the same positions as their MlsA2 counterparts. However, seven EryKS3 residues (Ala154, Lys155, Phe156, Val173, Ala230, Phe263 and Phe265) are replaced by other amino acids in MlsA2 KS (Fig S2). Except for Ala154 and Phe265, the substitutions are the same in all 16 mycolactone KS domains (Lys155Ala, Phe156Glu, Val173Met, Ala230Thr, and Phe263Thr). Ala154 is replaced either by Gly or Trp, and Phe265 is either conserved or replaced by Trp. These seven residues were therefore selected for mutagenesis of the EryKS3 domain, to determine their potential role in promoting broader substrate specificity.

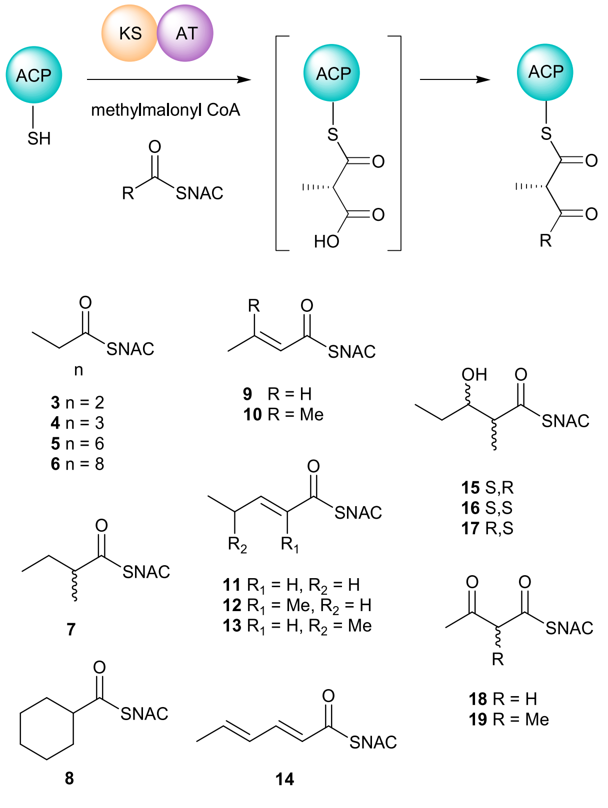

Fig. 2. Assay design and *N*-acetylcysteamine thioester substrates

Table 1. Percentage of EryACP3 carrier protein bearing Claisen condensation product after incubation with EryKS3AT3 or its mutants, methylmalonyl CoA and synthetic SNAC substrate. T indicates that only trace levels were detected (see SI for details).

	eryKS3AT3	$A_{154}G$	$A_{154}W$	$K_{155}A$	$F_{156}Q$	$V_{173}M$	$A_{230}T$	$F_{263}T$	$F_{265}W$
3	4	4	46	3	T	6	4	T	
4	3	6	83	6	7	4	2	23	
5			77	T	3			T	
6			13						
7	1	5	20	2	T	T	T	T	
8	2	2	72	T	5	2	1	4	
9			2						
10	6	17	91	16	18	11	8	10	
11		1	5						
12			2						
13			24						
14			33						
15	2	3	7	5	2	2	T	4	
16									
17			T	T	T				
18									
19									

To gain insight into specificity determinants in *cis*-AT KS domains, we used a sequence alignment of 199 domains to compute the sequence variability at a given position of a *cis*-AT KS domain, expressed as the percentage of sequences where the consensus residue is present (Figs. S3 and S8). This showed that for *cis*-AT KS domains the "dimer-interface loop" is the most prominently variable. Residues Ala154, Lys155, Phe156 chosen for mutagenesis in this study reside within this loop.

Mass spectrometric assay for *in vitro* ketosynthase activity

The catalytic competence of recombinant EryKS3AT3 has previously been monitored using a radio-TLC based assay involving radiolabelled *N*-acetylcysteamine (NAC) thioester **15**.[30] More recent studies on *trans*-AT KS domains have successfully used mass spectrometry to assay both self-acylation of the KS[15,16] and subsequent Claisen condensation.[17] We also chose a mass spectrometric method to directly monitor the formation of ketide-ACP condensation products, starting from an acyl-NAC thioester, recombinant EryACP3 and either methylmalonyl- or malonyl-CoA. Acyl-NAC thioesters are convenient surrogate substrates for EryKS3AT3 even though where K_m values have been reported they are 2-3 orders of magnitude higher than those of the corresponding acyl-ACP substrates.[10] First, we confirmed that when diketide NAC-thioester **15** was incubated with methylmalonyl-EryACP3 in the presence of EryKS3AT3, as described in the Supplementary Information, a new acyl-EryACP3 species was formed whose mass corresponded to that of the expected Claisen condensation product. A panel of acyl-NAC thioesters was then synthesised (Fig. 2) and assayed as substrates for Claisen condensation by KS3. This panel included acyl-thioesters of varying chain length; acyl-thioesters containing each of the functional groups routinely encountered during polyketide biosynthesis (β-keto-, β-hydroxy- and 2-enoyl-thioesters); and acyl-thioesters bearing an alkyl branch. For each substrate, the percentage of ACP bearing the respective ketide condensation product after 1h of incubation was measured (Table 1). Acyl transfer from SNAC thioesters to *holo*-EryACP3 was an observable background reaction, but this was not accelerated by the presence of EryKS3AT3, and is presumably the result of direct chemical thioester-thiol exchange. Substrates featuring α,β-unsubstituted thioesters were also susceptible to side reactions involving 1,4-conjugate addition of phosphopantetheine. Of the substrates tested, as well as the diketide thioester **15**, the alkanoyl thioesters **3** and **4,** the C-2 branched thioesters **7** and **8**, and the 2,3-unsaturated thioester **10** yielded modest amounts (2-6%) of β-ketoacyl-ACP species.

Different mycolactone KS domains efficiently carry out condensation with either malonyl- or methylmalonyl-ACP as extender units[25] so we also sought to evaluate the effect of the chosen active site mutations on the ability of EryKS3AT3 to catalyze condensation with malonyl-ACP. In fact, even native EryKS3AT3 has been reported to catalyse condensation with malonyl-ACP as the extender unit *in vitro*, albeit less efficiently than with the natural methylmalonyl-ACP substrate.[30] The malonyl-CoA-specific AT from MLS module 9 was cloned and expressed, and used to prepare malonyl-ACP3 (see Fig. S7) *in situ* from *holo*-EryACP3. EryKS3AT3 and a NAC thioester substrate were added to initiate condensation. Wild type KS3AT3 accepted the same NAC thioester substrates and gave similar yields of condensation product from both malonyl- and methylmalonyl-ACP (see Table S4). These results show that the KS3 does not discriminate between these two ACP bound extender units, consistent with previous studies.[31]

The point mutation Ala154Trp dramatically improves *in vitro* ketosynthase condensing activity against several substrates

Having established the reactivity of wild type EryKS3AT3 with this range of NAC thioester substrates and with two alternative extender units, the mutant EryKS3AT3 enzymes were tested using the same conditions (Tables 1 and S4, and Fig. S5). Most mutations were found to exert little effect on either substrate (SNAc or extender unit) specificity or the overall yield of the reaction, with two notable exceptions. First, the condensation activity was completely abolished in the EryKS3AT3 mutant F265W, for all substrates tested, although its acyl transferase activity remained intact (data not shown). This residue is modelled (Fig. S2) to lie close in space to the catalytic triad required for KS-catalyzed condensation, and although Trp is tolerated in this position in certain mycolactone KS domains, it may be that here the increased steric bulk of the tryptophan sidechain interferes with either initial acylation of the KS or the condensation reaction itself. In contrast, mutant Ala154Trp showed a markedly increased substrate tolerance, giving β-ketoacyl-ACP product from nearly all SNAC thioesters tested, with the exception of β-ketoacyl SNAC thioesters **18** and **19**. This mutant also showed a significant increase in turnover of substrates compared to the wild type enzyme. To confirm these preliminary results, side-by-side comparison of Ala154Trp with the wild type was repeated with the inclusion of an internal standard to confirm the stability of the ACP-bound Claisen condensation products.[18] Side reactions proved to be less problematic in these experiments and improved turnover was observed for both wild type and mutant

EryKS3AT3. However, Ala154Trp continued to be both significantly more promiscuous and a more effective catalyst. Detectable levels of condensation product could be observed for substrates **5** and **7** even for the wild type, but only the mutant gave condensation products from substrates **6**, **9** and **11** – **14** (examples shown in Fig. S5). Comparative time courses for the wild type and mutant were carried out using substrate **4**, and initial rates were extracted by fitting the data to an equation that allows derivation of initial rates from reaction progress curves[32] (Fig. S6). This confirmed that the initial rate with Ala154Trp is 4.5-fold greater than with the wild type.

The Ala154Gly mutant behaved similarly to the wild type enzyme, even though this substitution is found in certain mycolactone domains, so the observed effect is not simply related to the size of the side-chain at this position. The observed increase in both substrate tolerance and catalytic efficiency for Ala154Trp suggests an important role for this region of the active site. Analysis of the EryKS3AT3 crystal structure using Pymol (www.pymol.org) showed that at least local rearrangement of the active site would be necessary to accommodate a Trp side-chain in place of Ala154. This residue is located at the start of the "dimer-interface loop",[21] a region that shows a distinctive lack of sequence conservation in *cis*-AT KS domains (Fig S3), indicating a possible role in substrate selection. This region is only partly structured in the crystal structure of EryKS3AT3,[20] and it may be that in the Ala154Trp mutant this loop is significantly re-ordered. Not only does residue Ala154 lie within the active site close to the dimer interface, but it is also between two regions that cryo-EM studies have recently implicated in docking interactions between the KS and both its ‘upstream’ and intramodular ACP[28] (Fig. S1). The exact mechanism by which this mutation modulates KS specificity and KS:ACP binding interactions remains to be defined by structural studies, which are in progress. Meanwhile, this present finding mirrors an analogous finding of improved substrate tolerance made for a single active site residue substitution in an AT domain in the erythromycin PKS.[33] It provides important encouragement for further exploration of targeted mutagenesis of residues predicted to shape, or control access to, KS active sites, with a view to expanding the range of acyl-ACPs accepted.

Acknowledgements and references

This work was supported by project grants from the Biotechnology and Biological Sciences Research Council (BBSRC) U. K. to P. F. L. (BB/J007250/1), from the Wellcome Trust to R. W. B. (094252/Z/10/Z), and a Herchel Smith Postdoctoral Fellowship from the University of Cambridge to A. C. M.

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