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Jean-Malo Massicard, Claire Soligot, Kira J. Weissman, Christophe Jacob

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Jean-Malo Massicard,[a] Claire Soligot,[b,c] Kira J. Weissman*[a] and Christophe Jacob*[a]

A key goal of modular polyketide synthase (PKS) engineering is to alter polyketide stereochemistry. Here we report that exchanging whole PKS modules is a more productive approach than swapping individual ketoreductase (KR) domains for introducing rare 'A2' and 'B2' stereochemistry into model polyketides, and identify four modular 'biobricks' for such synthetic biology efforts.

The complex polyketide natural products of bacteria are heavily exploited in both human and veterinary medicine.[1,2] Their exquisite target specificity and high binding affinity[3] result from the density and defined stereochemistry of their diverse functional groups. In particular, hydroxyl substituents participate in directional hydrogen bonding interactions and act as attachment points for further functionality, while methyl and other acyl groups can impose important steric constraints. Together, these control elements impart conformational preferences to the large, typically macrocyclic molecules, giving them a defined shape while maintaining overall backbone flexibility.[4] This intimate link between molecular architecture and activity makes stereochemistry an attractive target for manipulation in polyketide analoguing efforts.

To this end, efforts have focused on genetically engineering the multienzymes responsible for building polyketide skeletons, the modular polyketide synthases (PKSs).[5] Remarkably, each step in PKS-catalysed chain assembly is carried out by a dedicated enzymatic domain: an acyl transferase (AT) selects an extender unit and transfers it onto an acyl carrier protein (ACP), upon which it is joined to the growing chain by a ketosynthase (KS) domain. The intermediate can then undergo stereospecific tailoring of the newly-established functionality at the α- and β-positions by ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains. It is thus possible, at least in principle, to modify the stereochemistry of a particular functional group by targeting the domain responsible for its formation. In this context, the KR domains are notable, as they carry out reduction of the β-keto groups formed upon chain extension to yield secondary hydroxyls of defined stereochemistry (3*S* or 3*R*, respectively, when C2 > C4; the stereochemistry is reversed when the priorities are inverted). Such KRs are referred to as A1 and B1.[6] Certain KRs also catalyse epimerisation at the adjacent α-methyl centre[7] generating the 2*S* configuration (A2 and B2 KRs, respectively).[8] Thus, in theory, engineering of type 2 KRs could result in modification at two adjacent stereocentres.

Indeed, knowledge of these different types of KRs has enabled attempts to modify polyketide stereochemistry by KR domain exchange within engineered, mini PKSs.[9–11] These systems include DEBS 1-TE[12] derived from the erythromycin PKS, in which the chain terminating TE has been fused to the end of DEBS 1, giving rise to a triketide δ-lactone incorporating two methyl and two hydroxyl stereocentres (**Fig. 1**). In earlier work, the A1 KR domain of the second chain extension module was substituted by a panel of B1, A2 and B2 KR domains.[9] While introduction of the B1 KRs resulted in the predicted product **3** (**Fig. 1**), only traces of the anticipated products **4** and **1** were obtained by swapping with A2 or B2 KRs, respectively.

In follow-up experiments,[10] we evaluated an extensive pool of A2 and B2 KRs. Globally, the nine tested A2-type KRs produced mixtures of epimerised and non-epimerised lactones (**4** and **2**, respectively), as well as substantial quantities of ketolactone **5**. Notably, however, two KRs (amphotericin (Amph) KRs 1 and 11) yielded **4** as the major product, and in respectable yield (7−14 mg/L). Discouragingly, all six B2 KRs produced at best traces of the expected epimerised and reduced lactone **1**, although the modified modules were still active for chain extension, producing ketolactone **5**. Two conclusions emerged from this work: i) the KR modular environment can influence the fidelity of stereocontrol (i.e. out of their native context, epimerising A2-type KRs can reduce non-epimerised chains); and, ii) B2 KRs exhibit low portability into an A1 modular context. These results prompted us to explore whether swapping module 2 of DEBS 1-TE for intact modules containing A2 and B2 KR from heterologous PKS systems (**Fig. S1**, ESI†), could improve both the absolute and relative yields of reduced lactones **1** and **4**.

[a] *Université de Lorraine, CNRS, IMoPA, F-54000 Nancy, France.*
[b] *Université de Lorraine, UR AFPA, USC 340 INRAE, F-54000 Nancy, France.*
[c] *Université de Lorraine, PASM, F-54000 Nancy, France.*

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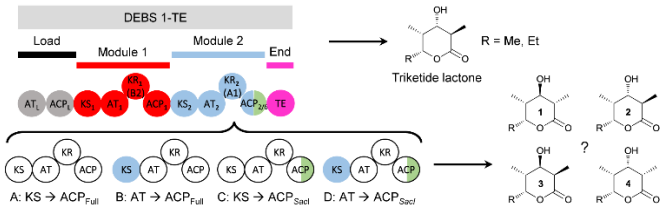

Fig. 1 Engineering strategies (A–D) used to replace module 2 of the model PKS DEBS 1-TE by heterologous modules containing A2 and B2 KRs. Key: AT, acyl transferase; ACP, acyl carrier protein; KS, ketosynthase; KR, ketoreductase; TE, thioesterase.

As target modules, we chose those harbouring four KRs tested previously[10] which gave a range of results: Amph[13] module 11 (M11) (A2 KR, predominantly **4**), oligomycin (Olm)[14] M5 (A2 KR, exclusively **5**), lankamycin (Lkm)[15] M1 (B2 KR, largely **5**, but also traces of **1**), and lasalocid (Las)[16] M7 (B2 KR, exclusively **5**). For the A2 KRs, we also tested bafilomycin (Bfm) M4 and M9[17] (and M4 and M9 of the closely-related hygrobafilomycin (HbaA) PKS[18]), and nystatin (Nys) M1 and M11 (**Fig. S1**, ESI†),[19] which had not previously been examined. We also studied one additional B2 KR-containing module, mediomycin (Med) M7a (**Fig. S1**, ESI†),[20] as this was one of the few novel modules of this type described in the literature when this project was initiated. The selected modules are present in different contexts: Amph/Nys M1 and M11, Olm M5 and Lkm M1 are linked covalently to an upstream module, analogous to the situation within the parental DEBS 1-TE system. In contrast, Las M7, Bfm/HbaA M4 and M9, and Med M7a are located at the N-termini of their respective subunits, and thus communicate with upstream modules non-covalently.[21] It was thus of interest to investigate the functional consequences of linking such modules covalently to an upstream module, as this question has not yet been explored comprehensively.[22–25]

PKS modules have been classically defined as running from KS to ACP, and encompassing all intervening domains (KS-AT-(DH-ER-KR)-ACP).[26] Many mono-module subunits contain precisely this complement of domains, notably including Las M7 and two other subunits from the Las system.[16] According to this definition, a module includes the KS and ACP domains which partner each other during chain extension. Module exchanges based on such classical boundaries thus preserve the key condensation interaction, while introducing a non-native ACP/KS chain transfer junction. This model has been challenged by phylogenetic analysis[20] which revealed co-evolutionary relationships between certain KSs and the upstream processing domains. This observation has led recently to a proposed redefinition of module borders (AT-(DH-ER-KR)-ACP-KS),[26,27] in which the KS is associated with the ACP domain from which it receives the extending chain. Indeed, it has been reported that ACP/KS interactions during intermodular substrate translocation are more important than substrate recognition in determining turnover efficiency, at least in chimeric synthases.[28] Furthermore, several published module swap experiments were only successful or produced substantial gains in yield, when employing modules with these redefined boundaries.[25,29,30] Nevertheless, module exchanges carried out in this way also generate a non-native interaction, that between the KS and ACP domains participating in chain extension.

Given the uncertainty surrounding which domains constitute a functional module and the degree to which non-native KS/ACP interactions impact on synthesis, we aimed to directly test multiple module boundaries (**Fig. 1**, **Table S1**). The first (**Fig. 1**, A) was to use classical modules, replacing DEBS 1-TE module 2 by KS-AT-KR-ACP tetradomain regions from heterologous PKSs. The upstream fusion point was chosen within a highly-conserved region at the N-terminus of the KS domains (PIAI motif), such that the native linker joining module 1 to module 2 was preserved (**Fig. S2**, ESI†).[22] In the second approach (**Fig. 1**, B), only the AT-KR-ACP tridomain of module 2 was replaced using a site at the conserved C-terminal end of the KS domain, thereby keeping intact the native DEBS ACP$_1$/KS$_2$ junction (**Fig. 1**). The chosen fusion site (**Fig. S2**, ESI†) was used previously in successful module swaps within DEBS 1-TE,[25] and is located in close vicinity to the recently exploited GTNAH motif (**Fig. S2**, ESI†) that permitted efficient AT swapping.[31] A subset of these experiments were, in addition, carried out with two versions of the ACP domains (**Fig. 1**), as the ACP in DEBS 1-TE is a hybrid between DEBS ACPs 2 and 6.[12] We thus opted to retain the hybrid character of the ACPs in certain experiments (**Fig. 1**, C and D) by carrying out exchanges up to the *Sac*I site at the ACP$_{2/6}$ junction (**Fig. S2**, ESI†), but in addition, using the complete heterologous ACP domains. In this second case, we used as the point of fusion the conserved C-terminal end of the ACP (**Fig. S2**, ESI†), thereby preserving the native ACP-TE linker.[32]

The starting point for engineering was the gene encoding for DEBS 1-TE within an *Escherichia coli*/*Streptomyces* shuttle vector pMU3-DEBS 1-TE,[10] which enables heterologous expression in a previously-described strain of *Saccharopolyspora erythraea*, JC2 (**Table S1**, ESI†).[33] To allow for exchange of module 2 of DEBS 1-TE with heterologous modules by transformation associated recombination (TAR) cloning in *Saccharomyces cerevisiae* strain 23.344c, pMU3-DEBS 1-TE was first modified by insertion of the URA3-CEN6-ARS4 sequence,[34,35] to furnish the *E. coli*/*Streptomyces*/*Saccharomyces cerevisiae* shuttle vector pMU3-DEBS 1-TE-URA3 (**Table S1**, ESI†). The target modules were then amplified by PCR (**Table S2**, ESI†) to incorporate suitable regions of homology with pMU3-DEBS 1-TE-URA3,[36] and co-transformed together with linearised pMU3-DEBS 1-TE-URA3 into strain 23.344c. Successful, scarless module exchange was then verified by diagnostic digestion and DNA sequencing. The module modified DEBS 1-TE constructs were transformed into *Sac. erythraea* JC2 by transconjugation with *E. coli* ET12567 (pUZ8002). The resulting recombinants were grown as described previously,[10] and the triketide lactone products were extracted and analysed by GC-MS.

In *Sac. erythraea*, the mini-PKS use propionate (**a**) and acetate (**b**) as the starter unit. Thus overall, eight reduced lactone products (**1a**/**1b**–**4a**/**4b**, **Table 1**) were possible in which the stereochemistry at carbons 2 and 3 varies (positions 4 and 5 are set by module 1, which was not altered in this experiment), as well as two 3-ketolactones (**5a**/**5b**). Assignment of product identity was based on comparison to synthetic standards (a 2.5:1 mixture of **1a** and **3a**, as well as **2a**) and biosynthetic standards (**1a**/**1b**–**3a**/**3b** and **5a**/**5b** from previously described strains JC2/pJLK25, JC2/pJLK30 and JC2/pJLK35[9] (**Fig. S3**, ESI†; all retention times are provided in **Table 1**)). Lactone **4** was assigned by elimination, as only four reduced products are possible. Product yields were quantified based on a standard curve generated using the synthetic lactones **1a**/**3a**, for which there was a linear correlation between peak area and product concentrations in the range of 5–200 mg/L (**Fig. S4**, ESI†). Three clones of each

construct were analysed (the average yields are summarised in **Table 2**, while a representative analysis of each construct along with a sample yield calculation are provided in the ESI†).

Table 1. Possible products resulting from exchanging module 2 of DEBS 1-TE for modules containing A2 and B2 KRs.

Catalytic events (Introduced module)	Product	Retention time (min)[a]	
		R = Et (a)	R = Me (b)
B2: Epimerisation + B-type reduction		14.9	13.9
A1: No epimerisation, A-type reduction		15.3	14.3
B1: No epimerisation, B-type reduction		15.6	14.6
A2: Epimerisation + A-type reduction		15.8	14.8
No reduction		14.1	13.1

[a]We observed a dependence of the retention time on product yields, with low quantities of lactone leading to variation of as much as 0.1 min. However, these discrepancies were never large enough to preclude conclusive assignment of stereochemistry.

Table 2. Yields of lactones resulting from introduction of modules containing A2 and B2 KRs into DEBS 1-TE.

Module introduced	Strategy	Yields (mg/L)[a]				
		1	2	3	4	5
Amph M11 (A2)	A		6.1 ± 0.5		36 ± 1	6 ± 1
	B		6.8 ± 0.7		20.2 ± 0.8	8.7 ± 0.4
	C		nd		nd	nd
	D		nd		nd	nd
Nys M1 (A2)	A		traces		9.4 ± 0.2	≃ 2
	B		traces		≃ 1.2	≃ 1.6
Nys M11 (A2)	A		≃ 2		18.9 ± 0.8	≃ 2.6
	B		≃ 4		9 ± 2	≃ 3.8
Olm M5 (A2)	A		nd		nd	14.6 ± 0.6
	B		nd		nd	6 ± 2
	C		nd		nd	nd
	D		nd		nd	nd
Bfm M4 (A2)	A		nd		nd	nd
	B		nd		nd	nd
Bfm M9 (A2)	A		nd		nd	nd
	B		nd		nd	nd
Hba M4 (A2)	A		nd		nd	nd
	B		nd		nd	nd
Hba M9 (A2)	A		nd		nd	nd
	B		nd		nd	nd
Lkm M1 (B2)	A	≃ 3.5		nd		≃ 2.7
	B	14 ± 1		traces		≃ 4.7
	C	nd		nd		nd
	D	nd		nd		nd
Las M7 (B2)	A	nd		nd		nd
	B	nd		nd		nd
	C	nd		nd		nd
	D	nd		nd		nd
Med M7a (B2)	A	nd		nd		nd
	B	nd		nd		nd

[a]Average yields and standard errors of experiments performed in triplicate are reported. nd indicates that the lactone was not detected.

The first important result is that none of the constructs incorporating hybrid ACPs was functional (**Table 2**). This result makes the reported strong activity[9,12] of parental DEBS 1-TE and certain of its derivatives surprising, but clearly underlines the need to keep ACP domains intact in module swap experiments. In our previous work, among all A2 KRs tested, Amph KR_{11} gave both the highest relative and absolute yields of the desired methyl-epimerised and reduced product **4** (17 mg/L total lactone, 82.4% **4**, 17.6% ketolactone **5**). As hoped, exchanging a larger portion of the module resulted in substantially improved amounts of lactone **4**, albeit at the expense of relative yield (**Table 2**). Using the classical module boundaries (tetradomain KS-AT-KR-ACP; Amph M11-A), the overall yield of lactone increased almost 3-fold to 48.1 mg/L, of which 75.0% was lactone **4** (36 mg/L). The remaining product was evenly split between the ketolactone **5** observed previously (12.5%), and reduced, but non-epimerised lactone **2** (12.5%). Thus, the total amount of reduced lactone of either stereochemistry was also boosted relative to the KR swap experiment (87.5% vs. 82.3%). As in our previous work,[10] no products (**1** or **3**) corresponding to incorrect B-type reduction were observed. Using the alternative module boundaries (tridomain AT-KR-ACP; Amph M11-B) produced an approximately 2-fold increase in total lactone yield compared to the KR exchange (35.7 mg/L), but resulted in a greater proportion of **2** (19.0%), as well as ketolactone **5** (24.4%).

In keeping with the close homology between the Amph KR_{11} and Nys KRs 1 and 11 (84.1% and 69.3%, respectively), exchanging partial or whole Nys KR-containing modules resulted in the desired product **4**, and of the two modules, the Nys M11 gave the higher yields (**Table 2**). These results are reminiscent of swapping Amph KRs 1 and 11 into DEBS 1-TE, wherein Amph KR_{11} was superior,[10] showing that KRs/modules sourced from later in the assembly lines operate better within a module 2 context. Again, the full module swap in each case produced both higher overall and relative yields of **4**, compared to the alternative module boundaries (**Table 2**; Nys M1-A, Nys M11-A). Stereochemical fidelity of both Nys modules was also moderately stronger than for Amph KR_{11} (traces and 8.5% **2**, respectively). Taken together, these results demonstrate that heterologous KR function is improved by maintaining a native modular context, but that substrate specificity is nonetheless compromised, as the non-epimerised chains were also reduced. We attribute this loss of fidelity to the fact that the KRs were acting on chains which differ in both length and functionality to their native substrates. By contrast, the downstream TE has been shown to favour the α,β-stereochemistry present in **4** for both hydrolysis and ethanolysis,[37] and thus is unlikely to have biased the product distribution towards lactone **2**. Of the remaining modules containing A2 KRs, only Olm M5 was functional, yielding ketolactone **5** (**Table 2**; Olm M5-A, B). In addition, the **5** yields of 14.6 mg/L and 6 mg/L, respectively, for the full and partial swaps, were substantially lower than the amount of **5** produced by the corresponding KR exchange (52 mg/L[10]). Thus, not only did Olm KR_5 fail to act, as observed previously, but the module swapping strategy further reduced module 2 chain extension activity. It is nonetheless notable that exploiting the classical boundaries to exchange the module again produced superior results.

In the case of the B2 KR-containing modules, only exchange of Lkm M1 produced the desired reduced and epimerised lactone **1**. Nonetheless, the yields (**Table 2**) were substantially improved relative to the corresponding KR swap, for which **5** predominated (6 mg/L) and only traces of **1** were observed. In contrast to the A2 KR-containing modules, higher relative and overall yields of **1** were obtained by swapping the AT-KR-ACP tridomain instead of the full module (18.7 mg/L total lactone, 75% **1** vs. ca. 6.2 mg/L, 56% **1**; **Table 2** (Lkm M1-B, A respectively)), although traces of the reduced, but non-epimerised lactone **3** were also observed. **3** was not present in extracts of the full module swap (Lkm M1-A), suggesting that Lkm KS_1 boosted the overall stereochemical fidelity of the module. However, its presence also led to reduced product yield, possibly due to a lower efficiency of intermodular chain transfer.[28,38]

None of the other module exchanges (A2-type Bfm M4/9 and HbaA M4/M9 and B2-type Las M7 and Med M7a) in either configuration yielded product (**Table 2**), despite the fact that in our previous work, swapping in Las KR_7 produced a hybrid module 2 functional for chain extension (8 mg/L of **5**[10]). In the case of the full module replacements, this failure is presumably due to lack of chain translocation between DEBS module 1 and the inserted module, while in the case of the AT-KR-ACP tridomain swaps which preserved the native DEBS ACP_1/KS_2 interface, the problem likely arose at the stage of the condensation reaction catalysed between DEBS KS_2 and a heterologous ACP domain.

In conclusion, the work reported here establishes module exchange as a superior strategy to KR swapping to access polyketides incorporating unusual A2 and B2 stereochemistry. In this context, we have notably identified three A2-type and one B2-type modules which give both high overall and relative yields of the desired products, making them valuable additions to a PKS engineering toolbox. The data are, on the other hand, mixed in terms of which group of domains defines a functional model.[26,27] Although using classical module boundaries (KS-AT-KR-ACP) worked best for the A2-type modules, the alternative tridomain (AT-KR-ACP) gave better results for the B2-type module. Evidently, the relative negative effects of non-native chain transfer and chain extension interactions on chimeric PKSs can differ depending on the identity of the source modules. It is also notable that all of the functional modules are covalently joined to upstream modules within their native subunits. In such contexts, they have no 'choice' of donor acyl-ACP domain, and thus apparently have not evolved to discriminate strongly against alternative acyl-ACP substrates. In contrast, all of the non-functional swapped modules are located at the N-termini of their respective polypeptides. In these cases, the intermolecular nature of substrate delivery evidently imposes stricter specificity constraints on the module, both in terms of protein-protein (ACP/KS) and domain-substrate interactions.[38] Taken together, these findings argue that modules which are internal to subunits are intrinsically more adapted to relocation by genetic engineering into novel covalent contexts than their N-terminal counterparts.

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Conflicts of interest

There are no conflicts to declare.

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