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Engineering enzymatic assembly lines to produce new antibiotics

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Numerous important therapeutic agents, including widely-used antibiotics, anti-cancer drugs, immunosuppressants, agrochemicals and other valuable compounds, are produced by microorganisms. Many of these are biosynthesised by modular enzymatic assembly line polyketide synthases, non-ribosomal peptide synthetases, and hybrids thereof. To alter the backbone structure of these valuable but difficult to modify compounds, the respective enzymatic machineries can be engineered to create even more valuable molecules with improved properties and/or to bypass resistance mechanisms. In the past, many attempts to achieve assembly line pathway engineering failed or led to enzymes with compromised activity. Recently our understanding of assembly line structural biology, including an appreciation of the conformational changes that occur during the catalytic cycle, have improved hugely. This has proven to be a driving force for new approaches and several recent examples have demonstrated the production of new-to-nature molecules, including anti-infectives. We discuss the developments of the last few years and highlight selected, illuminating examples of assembly line engineering.

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Introduction

Articles addressing antimicrobial resistance (AMR) with titles such as '*Antibiotic apocalypse: doctors sound alarm over drug resistance*' (The Guardian), regularly hit the news headlines and are no longer limited to the scientific community. Amongst the many solutions discussed on the issue of AMR [1], a repeatedly stressed source for discovering new therapeutic agents are specialised metabolites (SM) [2,3]. SMs are obtained from living organisms and comprise structurally diverse and bioactive molecules with low molecular weight (<3000) [4] which occupy chemical property space beyond Lipinski's rule of five [5].

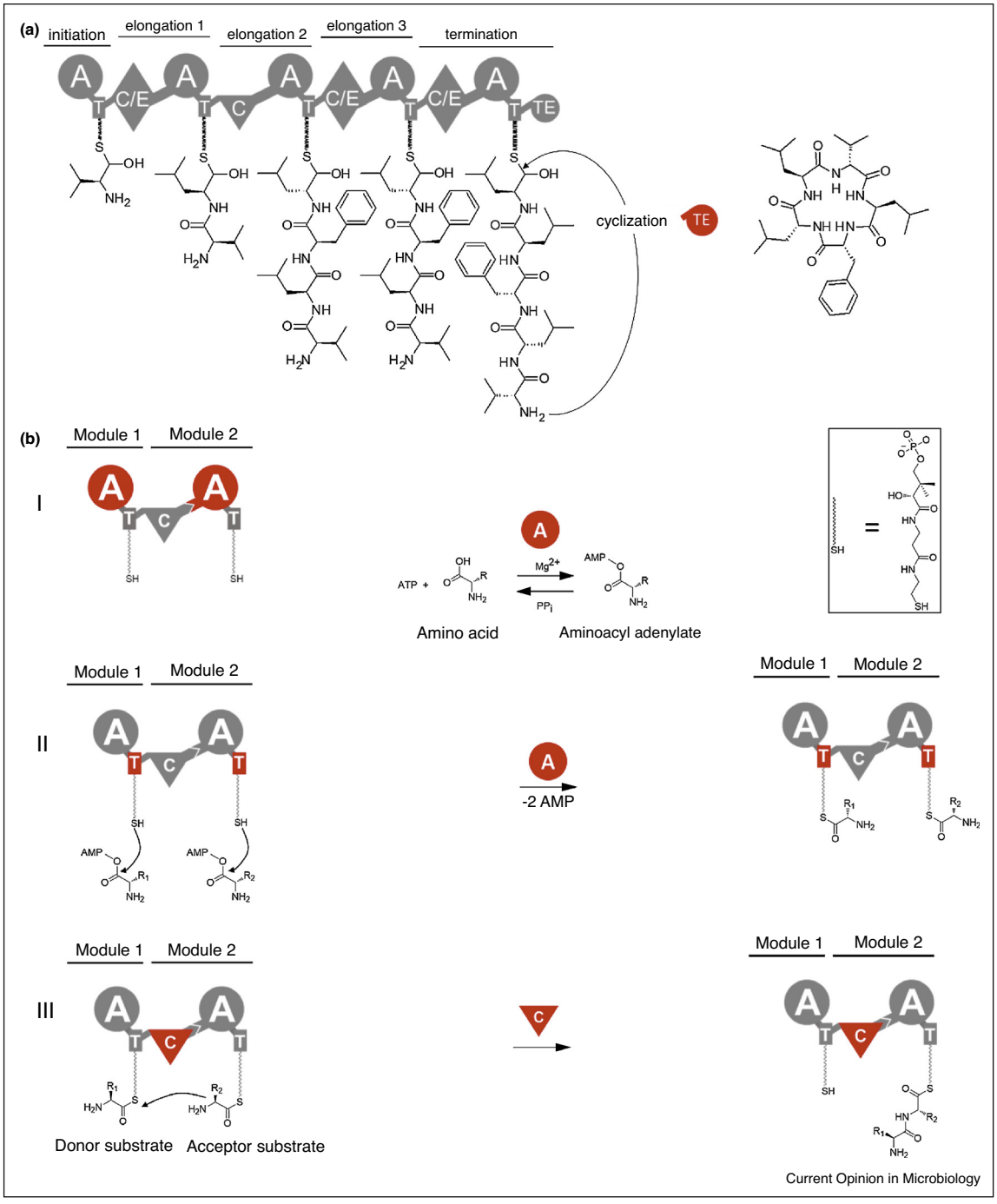
Recent advances in next-generation DNA sequencing methods [6], in conjunction with genome [7] and structure mining [8–10] approaches, including mass spectrometry-based proteomics [11], have guided the identification and isolation of novel SMs with antimicrobial activity. In addition, a high-throughput screening approach for the discovery of SM antibiotics was recently reported [12], while chemoinformatic approaches [13], and enhanced methodology for the identification of antibiotics [14] and their producing strains have been developed [15]. Notably, technological advances in metagenomics [16] and *in situ* cultivation [17] led to the identification of two promising new classes of antibiotics, the malacidins (metagenomic acidic lipopeptide antibiotic-cidins) and teixobactins, respectively.

To become drugs, SMs are often chemically modified to fine tune their biological activities and/or improve biophysical properties [18]. Because of their complex structures, many clinical SM derivatives have been created by means of semi-synthesis, for example, azithromycin [19]. Because of technical and chemical limitations, such modifications are often confined to a few synthetically accessible functional groups and leave the backbone structure untouched. In contrast, bioengineering offers an alternative approach to alter SM backbones and access a wider range of structural diversity.

The complex chemical structures and enormous pharmaceutical potential of the non-ribosomal peptides (NRPs; e.g. teixobactin) [17] and polyketides (PKs; e.g. avermectin) [20] make these SMs key targets for bioengineering. They are synthesised on large modular non-ribosomal peptide synthetases (NRPS) [21] (Figure 1) and polyketide synthases (PKS) [22] (Figure 3) respectively, using a conserved multiple-carrier thiotemplate mechanism [23]. They use peptide bond formation (NRPS), Claisen-type condensation reactions (PKS) or combinations thereof (NRPS/PKS hybrids) to build larger molecules from simple building blocks [24,25].

Commonly, the number of modules in a NRPS or PKS corresponds directly to the number of monomers incorporated into the associated SM, and the arrangement of the modules directly follows the SMs' primary sequence because synthesis proceeds in a colinear fashion, enabling structural predictions [8,26]. Once discovered, their

Figure 1



Introduction to modular non-ribosomal peptide synthetases.
(a) The modular architecture of NRPSs, exemplified by the GameXPeptide-Synthetase (GxpS). A NRPS module is defined as the catalytic unit responsible for the incorporation of one-specific building block into the growing peptide chain and associated functional group modifications. Enzymatic activities of a canonical module reside in the adenylation (A), thiolation (T), and condensation (C) domains. **(b)** Firstly, amino acids (AAs) are activated through the activity of an A-domain (I). The energy derived from ATP hydrolysis is used to form an aminoacyl-adenylate intermediate. Then the AA is loaded onto the thiol of the pantetheine cofactor of the T domain (II). Consecutively activated AA on T domains are then joined by a C-domain that catalyses peptide-bond formation and transfers the upstream AA or peptide to the downstream substrate (III). Finally, most NRPS termination modules harbour a TE (thioesterase) domain, usually responsible for the release of linear (transfer to a water molecule), cyclic or branched cyclic peptides (amide or ester linkage). In addition, a cyclization (Cy) domain instead of a C-domain or a terminal condensation (Cterm) domain in place of a TE-domain can be present. Modification domains include epimerization (E), N-methylation (MT) or oxidation (Ox) domains.

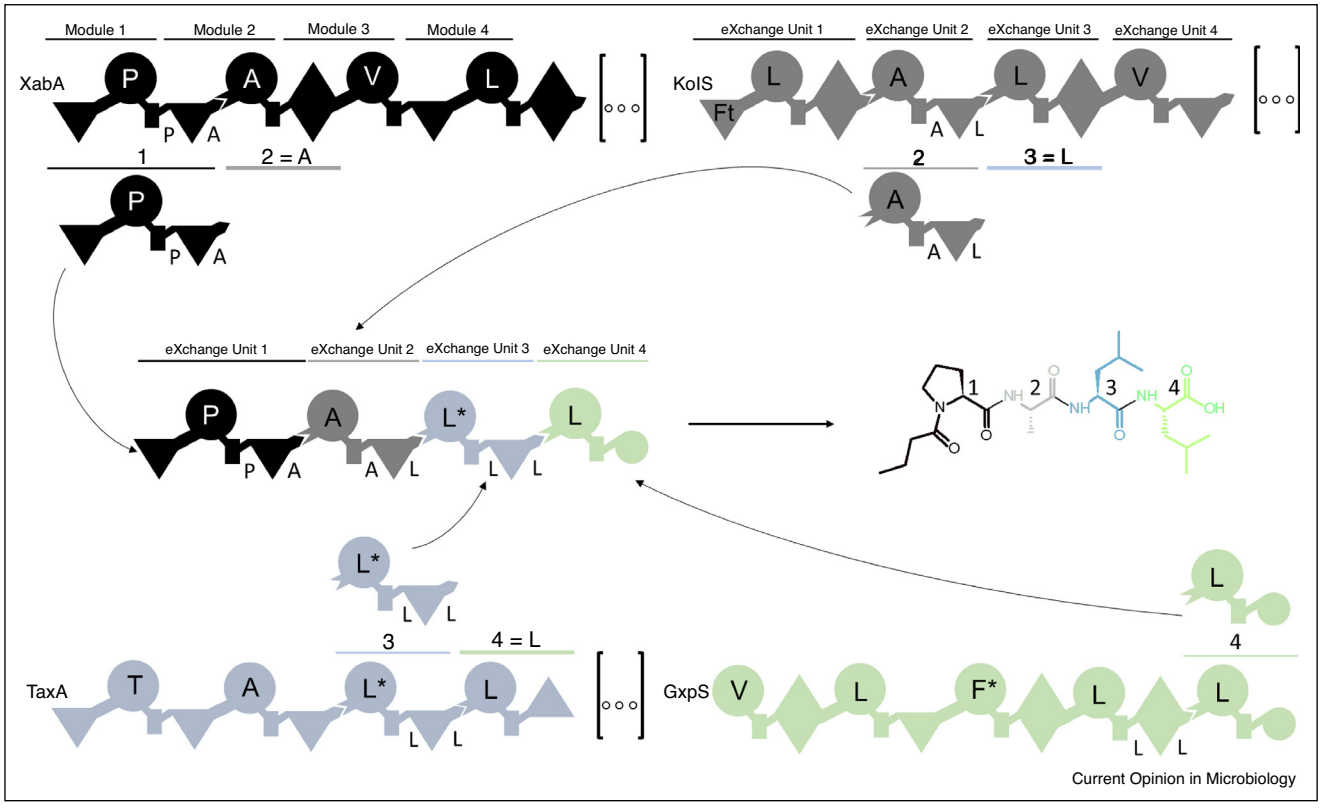
modular architecture immediately suggested the possibility for rational manipulation [27]. This was realised for the first time in 1995, for both NRPS [28] and PKS [29]. Since then the synthetic biology community has envisaged using these enzymes like a molecular toolkit to modify and create novel SMs in a tailormade fashion. Unfortunately, many attempts to achieve assembly line pathway engineering have yielded biosynthetic machineries
that are either greatly impaired in their activity or non-functional [30].

Much of the fundamental biochemistry regarding mega-synth(et)ases, in addition to crystal structures of domains, didomains, and even whole modules, had been solved by the early years of the new millennium [31]. Despite this, innovation in their bioengineering slowed significantly. However, due to recent technological advances [32,33], our structural as well as conformational knowledge of these enzymes has improved significantly [34•,35]. Valuable insights into the high structural flexibility, and the importance of inter-domain communication, provided a new impetus for a series of novel strategies to engineer modular biosynthetic pathways [36–41]. Recent publications have demonstrated the production of new-to-nature SMs, including antimicrobials, and we highlight here selected key developments from the last 2–3 years. The principles of NRPS and PKS biosynthesis as well as key enzymatic domain abbreviations are introduced in Figures 1 and 3, respectively.

NRPS: challenging dogma

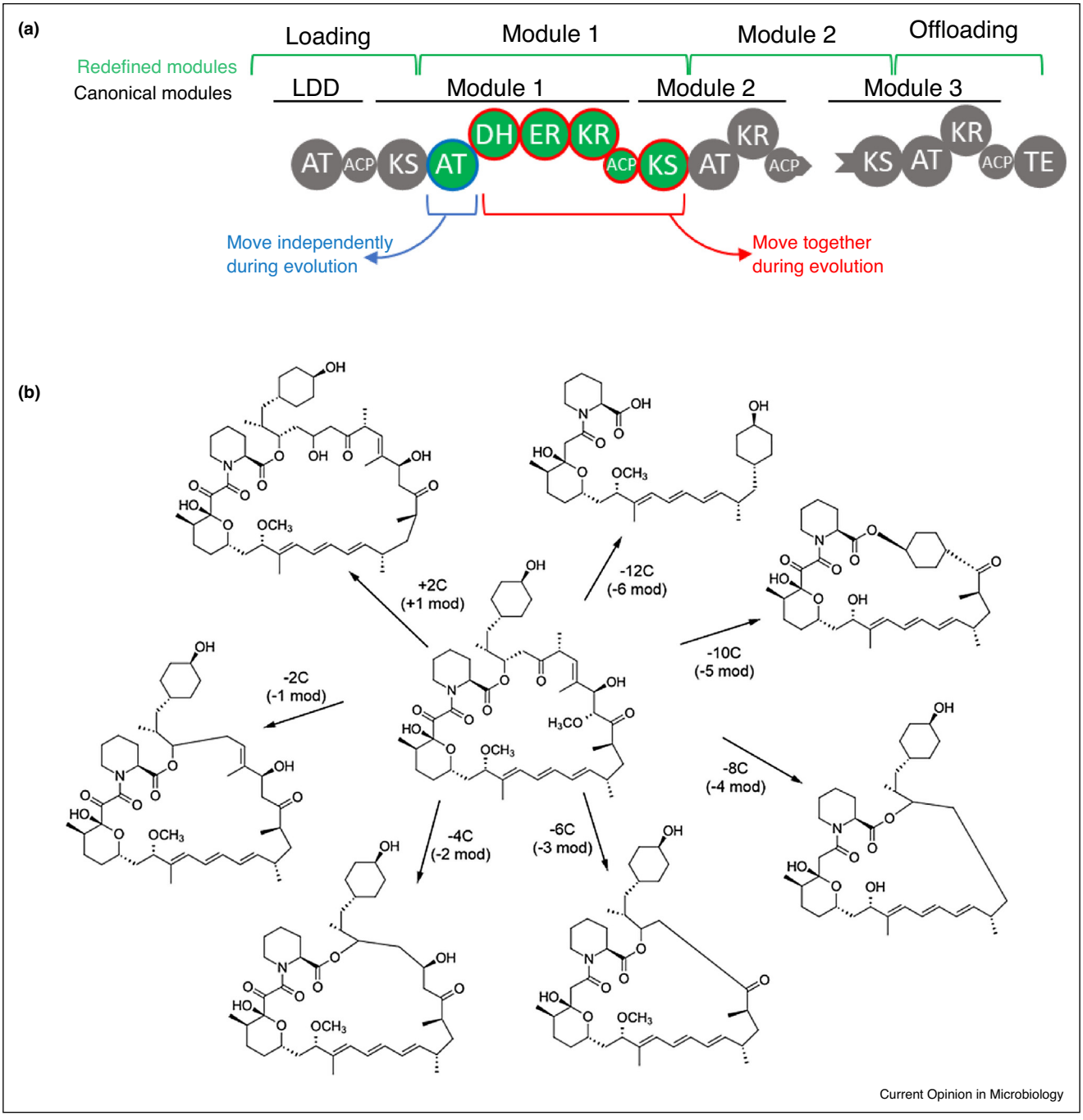
Mega-synth(et)ase engineering involves numerous challenges, including the impact of gatekeeper domains to prevent the incorporation of incorrect extender units into the final product, or limited structural information making it difficult to predict functional protein-protein interactions. Previously, researchers have employed three general strategies to achieve this: (**1**) substitution of the A-domains or paired A–T domains activating an alternative substrate; (**2**) targeted mutagenesis of the substrate binding pocket of an A-domain; (**3**) substitutions that treat C–A or C–A–T domain units as inseparable pairs [30].

Figure 2



The eXchange Unit.
Schematic representation of NRPS recombination rules using XUs from the Xenoamicin (XabA), Kolossin (KolS), TaxIIIaid (TaxA), and GameXPeptide (GxpS) synthetase considering the C-domain specificities. Symbols used for domain assignment are: A, large circle; T, rectangle; C and formyl transferase (Ft), triangle; C/E, diamond; TE, C-terminal small circle. Amino acid specificities of the A-domains, C-domains and C/E-domains are indicated by capital letters using the standard one letter AA abbreviations. Promiscuous domains are indicated by '*'.

Figure 3



Engineering polyketide synthases.
(a) Truncated representation of 6-deoxyerythronolide B synthase. Like NRPS A-domains, the PKS acyltransferase (AT) domains select malonyl-derived coenzyme A (CoA)-linked extender units. The resulting malonate derivatives are transferred to acyl carrier proteins (ACP) which function like NRPS T-domains, and ketosynthase (KS) domains act like NRPS C-domains. PKSs also have additional tailoring domains present to modify the PK-chain, that is, ketoreductase (KR), dehydratase (DH), enoylreductase (ER), and methyltransferase (MT) domains. The revised definition of modules (green) as well as co-evolving domains (blue, red) are highlighted. **(b)** Rapalogs, a diversity-oriented library of rapamycin derivatives gained by laboratory-scale evolution, termed 'accelerated evolution'. Forced recombination events of the highly homologous rapamycin pathway lead to the loss of modules (mod) 1–6 or the addition of a second copy of module 13.

A key dogma, leading to strategies (**1**) and (**2**), says that A-domains represent the main gatekeeping enzymes [42]. Recent insights are challenging this view and explain why strategies (**1**) and (**2**) can only be reliably applied within initiation modules lacking an upstream starter C-domain (C_{start}) [43,44]. In addition, termination domains represent often overlooked bottlenecks. For example, impaired reprogrammed NRPS assembly lines could be 'cured' by replacing TE-domains with 'common internal' C-domains to produce linear-peptides, cyclic-peptides, and depsi-peptides [45••]. Recently, Yu *et al.* utilised different C_{term}-domains of fungal iterative NRPSs to produce new cyclic peptide derivatives of different chain length [46].

Engineering the specificity of individual A-domains (**1**) from initiation modules represents a proven method for creating structural diversity [47–49]. Niquille *et al.* presented the first high-throughput assay to selectively alter the promiscuous L-Phe activating starter A-domain from the tyrocidin NRPS (TycA) to genuinely accept the β-amino-acid residue (*S*)-β-Phe [50•]. A smart 'click'-chemistry mediated fluorescent yeast cell surface display assay [51] for catalytic activity in combination with FACS allowed substrate walking from L-Phe to (*S*)-β-Phe via screening >1 million variants. With a (*S*)-β-Phe specific A-domain in-hand, the authors showed that scalable amounts of backbone-modified peptides could be produced *in vitro* (~1 mmol) and *in vivo* (~100 mg L^{-1}). Although the initiation module of TycA was targeted, to prevent proofreading of upstream C-domains, the strict specificity of the TE-domain for the first amino acid resulted in hydrolytic off-loading of the modified peptide rather than cyclic dimer formation. This strategy for NRPS domain evolution is very promising and similar approaches could be leveraged to optimise biosynthetic bottlenecks.

Another convention of NRPS engineering has been the inseparability of C–A domains (strategy **3**). The origin of this lies in the canonical definition of a module (C–A–T). In 2008, when the first crystal structure of a full-length termination module (C–A–T–TE) was published (PDB ID: 2VSQ) [31], the convention became dogma. This outstanding work delivered valuable insights into the high structural flexibility of NRPSs and the importance of inter-domain communication that must be maintained during NRPS engineering. Yet, this snapshot of the catalytic cycle led to the premature conclusion that C–A di-domains form a stable workbench that should not be separated [34•,52]. Recently, Bozhuyuk *et al.* took advantage of additional structural data and challenged the convention by introducing a rule-based mix-and-match strategy (Figure 2) [45••]. The NRPS C–A linker was a prime target for swapping A–T–C units, denoted as eXchange Units (XUs). By combining XUs from 15 NRPSs the authors were able to reconstitute naturally available peptides, peptide derivatives, and generated new-to-nature peptides *de novo* in good yields. The most important consideration, and therefore restriction, of this strategy is the substrate specificity of the downstream C-domain, which must be respected. The Mueller group also utilised A–T–C XUs to successfully modify lipopeptide BGCs in *Myxococcus xanthus*, suggesting their general applicability [53], but the downstream C-domain restriction calls for a very large number of available building blocks if new-to-nature NRPs are to be designed in appreciable numbers.

To access a high degree of structural diversity in good yield, a recent trend in NRPS engineering utilises evolution-guided strategies [47,54]. In the context of full-length modular NRPSs, the Abe lab successfully accomplished the manipulation of two antimycin-type cyclic depsi-peptides, both derived from a NRPS/PKS hybrid synthetase, to generate a set of derivatives in practical yields [55]. Another illuminating example was published by Meyer, Steiniger *et al.* [56•]. Engineering of iterative fungal cyclo-depsi-peptide pathways enabled the biosynthesis of enniatin and beauvericin derivatives by combining highly structurally and biochemically related NRPSs. The new peptide derivatives showed up to 12× increased activity against *Leishmania donovani* and *Trypanosoma cruzi* compared to the reference drugs miltefosine (Miltex™) and benznidazole (Rochagan™), respectively. Of important note was the ability to produce new-to-nature peptides at an industrially relevant scale (1.3 g L^{-1}).

PKS engineering: from rational engineering to accelerated evolution

In general PKS engineering faces the same challenges and applies the same design principles as, NRPS engineering. Common approaches for PKS engineering have involved the exchange of whole modules or individual domains, especially AT domains [57]. Although early attempts reported mixed results, the Keasling lab comprehensively analysed interdomain linkers in PKSs to define domain boundaries and provide guidelines for replacing AT-domains [58•]. Using monomodular PKSs as a model, the new highly conserved AT-domain boundaries were utilised to great effect, producing short-chain ketones without significant loss of activity for the hybrid enzymes. More recently Kalkreuter *et al.* applied the newly defined AT-boundaries to enable expansion of AT substrate to include non-natural extender units and exemplified this by targeting the final two modules of the pikromycin PKS (PikAIII, PikAIV) [59]. Initially this resulted in inactive or highly impaired chimeras. By further engineering the specificity of both modules, and utilising *in vitro* assays of bimodular reactions, they revealed that the KR-domains and KS-domains were more substrate-permissive than respective AT-domains. To take advantage of this promiscuity, cumulative AT active site mutagenesis, guided by homology modelling

and molecular dynamics simulations, resulted in robust yields of a pikromycin derivative with two non-natural extenders. The results of these two studies suggests there is, currently, no general method to engineer AT-domains, and that the reported successful attempts represent tailored, non-transferable solutions.

Although AT-domain swaps [58•] and active site mutagenesis [59] can be suitable for PKS engineering, the proofreading activity of downstream domains may limit the use of these approaches. This issue became apparent when the Keasling group introduced heterologous reductive loop swaps (KR/ER/DH) from various PKSs, as well as a TE-domain, into the borrelidin PKS module 1 to biosynthesise adipic acid [60]. Mass spectrometry-based Acyl-ACP intermediate analysis revealed an unexpected bottleneck at the dehydration step. Eventually this limitation was overcome by introducing a carboxyacyl-processing DH-domain, but the results once again highlighted that many caveats must be considered when engineering these systems, including the proof-reading activity of KR domains [61], especially in the context of full length multi-modular PKSs.

Gatekeeping domains are crucial for organisms to ensure biosynthesis of the desired SM, but such proofreading mechanisms can lead to bottlenecks of new SM production. Hansen *et al.* recently identified the pikromycin (Pik) AIII-TE domain to be of limited substrate flexibility and a key catalytic bottleneck in processing non-natural substrates [62]. Molecular dynamics simulations and quantum mechanical comparison revealed that introduction of a single mutation at the key active site residue (Ser148Cys) in PikAIII-TE significantly increased the substrate flexibility, enabling the production of diastereomeric macrolactones [63]. Similarly, the Leadlay group showed that a single residue change (Ala154Trp) in the KS3 of the erythromycin PKS led to an emphatic increase in turnover of a range of substrates [64]. Identifying and overcoming bottlenecks is very useful to fine tune tailored SM production, but to access a wider chemical space more disruptive bioengineering approaches are necessary.

To overcome PKS reprogramming issues an understanding of biosynthetic gene cluster evolution might provide a rationale for reprogramming assembly line machinery. For example, Zhang *et al.* challenged a long-standing paradigm in modular PKS, the definition of a module (Figure 3a). They suggested that for module swapping, the canonical module KS-AT-(DH/KR/ER)-ACP should be altered to AT-(DH/KR/ER)-ACP-KS [65••]. Bioinformatic analysis of highly homologous but functionally diverse domains from four giant aminopolyol producing PKSs demonstrated evolutionary relationships between equivalent domains. As also suggested previously [66], they proposed that AT domains move independently during assembly line evolution, whereas the β-processing domains, ACPs, and downstream KS-domains should be considered as a unit, and swaps that conserve the (DH-ER-KR)-ACPn-KSn+1 relationship would facilitate rational modular PKS engineering. Similar intermodular relationships can also be observed in the evolutionary analysis of several *trans*-AT PKSs [67,68], suggesting the generality of this mechanism during the evolution of PKS genes through recombination.

Stochastic approaches to PKS engineering can also lead to new SMs [69]. An illuminating example utilises a homologous recombination-based method to mimic evolutionary pressure, resulting in the accelerated evolution of modular PKS genes in which multiple modules were deleted, added, or replaced leading to a diversity orientated library of new rapamycin derivatives (rapalogs) with altered biological activities in high yields (Figure 3b) [70••]. In contrast to rapamycin, several of the rapalogs were devoid of immunosuppressive activity but retained potent inhibition of FKBP12 like enzymes including macrophage infectivity potentiator proteins (MIPs), validated virulence factors of Gram-negative pathogens. One rapalog was shown to increase the survival of human macrophages infection by the pathogen *Burkholderia pseudomallei*. Detailed sequence analysis of the recombinant genes revealed junction sites representing non-canonical module boundaries, and recombination 'hot-spots' were identified within KS and AT domains and in the linker upstream of ACP domains. 'Non-rational' strategies like this in combination with in depth comparative sequence analysis may lead to new design principles that eventually support novel rational engineering strategies. Further development of this method in combination with high throughput screening (e.g. fluorescence-activated cell sorting; FACS) could be used to generate structurally diverse SM-like libraries to identify novel antimicrobial entities.

Concluding remarks

Although landmark achievements in chemical and structural biology [34•,35] have advanced our understanding of modular SM pathway mechanisms, nature's proven ability to 'engineer' assembly-line enzymes remains difficult to recapitulate in the lab. However, the ever-increasing amount of genomic data, as well as the development of non-rational [70••] and high-throughput methods [50•], have enabled the development of novel strategies, especially when highly similar and iterative NRPS, PKS, or hybrids thereof are combined [45••,55,56•].

The ability to rationally swap modules and domains in assembly-line pathways has long been a 'holy grail'; with the redefinition of PKS modules, experiments based on evolutionary principles, and the identification of catalytically and structurally exchangeable units in NRPSs, this goal may soon be achievable [45••,65••]. Nevertheless, caution is advised, and these disruptive new insights must

be extensively evaluated, as future developments can be easily hampered when premature assumptions become dogma. To avoid this, we advocate the publication of unsuccessful or negative results, and an annual review on this subject would prove invaluable in evaluating the generality of recent developments. Insights gained could be used to delineate future guidelines, develop scoring methods to predict the capability of building blocks, and to identify and clarify bottlenecks. The authors call upon colleagues for concerted action in this direction, possibly publishing their negative data in an annual 'inglorious' review of assembly-line chemistry and biology. We learn valuable lessons from mistakes, and failure only serves to make us stronger.

Conflict of interest statement

BW is a board member and shareholder of Isomerase Ltd. (Cambridge UK).

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- •• of outstanding interest

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