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REVIEW

Progress challenges and opportunities for the re-engineering of *trans*-AT polyketide synthases

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Abstract Polyketides are a structurally and functionally diverse family of bioactive natural products that are used extensively as pharmaceuticals and agrochemicals. In bacteria these molecules are biosynthesized by giant, multi-functional enzymatic complexes, termed modular polyketide synthases (PKSs), that function in assembly-line like fashion to fuse and tailor simple carboxylic acid monomers into a vast array of elaborate chemical scaffolds. Modifying PKSs through targeted synthase re-engineering is a promising approach for accessing functionally-optimized polyketides. Due to their highly mosaic architectures the recently identified *trans*-AT family of modular synthases appear inherently more amenable to re-engineering than their well studied *cis*-AT counterparts. Here, we review recent progress in the re-engineering of *trans*-AT PKSs, summarize opportunities for harnessing the biosynthetic potential of these systems, and highlight challenges that such re-engineering approaches present.

Keywords Combinational biosynthesis · Enzyme engineering · Natural products · Polyketide synthase · Synthetic biology · *trans*-Acting AT polyketide synthase

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Introduction

Polyketides constitute an extraordinarily large and diverse group of secondary metabolites that are produced extensively by terrestrial and marine microorganisms and plants (Weissman 2009). Due to their potent biological activities, these molecules have found widespread application in human and veterinary medicine and agriculture, and include the broad-spectrum antibiotics, erythromycin and tetracycline, the anticancer immunosuppressant, rapamycin, and the cholesterol-lowering statins (Marinelli 2009). In bacteria, complex polyketides are biosynthesized by giant (up to ~10 MDa) multi-component megaenzymes of the type 1 modular polyketide synthase (PKS) class, in which the catalytic apparatus responsible for product assembly is organized into discrete multi-domain modules housed within large polypeptide chains (Fischbach and Walsh 2006). Biosynthesis in these systems proceeds via the sequential addition of coenzyme A (CoA)-derived extender units to an initial starter template, with each module responsible for the extension of the growing product chain by a single acyl monomer (Keating and Walsh 1999). Minimally, a PKS-extension module comprises an acyltransferase (AT) domain for extender unit selection and loading, an acyl carrier protein (ACP) for extender unit tethering via a covalent linkage, and a ketosynthase (KS) domain, catalyzing the decarboxylative Clasien condensation of the ACP tethered extender unit with the acyl thioester of the product

chain (Hertweck 2009). Frequently, the minimal AT–ACP–KS module architecture is elaborated to include additional functional domains that dictate the degree of β-ketoacyl reduction, e.g. ketoreductases, dehydratases and enoyl reductases. Modular PKSs share many functional similarities with mammalian fatty acid synthases (FASs), though their ability to use a selection of starter and extender units and to vary the oxidation state of incorporated β-keto groups generates a significantly greater degree of chemical diversity.

Since their initial discovery in soil-dwelling actinomycetes (Cortes et al. 1990), modular PKSs have been the subject of considerable genetic and biochemical investigation (Fischbach and Walsh 2006; Hertweck 2009; Weissman 2009). Of particular significance has been the establishment of so-called 'collinearity rules', linking synthase nucleotide sequence to polyketide structure (Minowa et al. 2007; Callahan et al. 2009), an advance that has proved instrumental in the prediction, identification, and manipulation of modular synthases. Recently, however, detailed analysis of genome sequencing data from more unusual bacterial taxa has resulted in the identification of a novel family of modular PKSs whose biosynthetic frameworks do not adhere to the established collinearity rules of the paradigm actinomycete modular synthases. These novel systems, termed *trans*-AT PKSs, are comprised of biosynthetically disparate enzymatic domains that function in concert within a unified synthase scaffold (Piel 2010). The functional disparities between canonical actinomycete *cis*-AT type systems and *trans*-AT synthases appear to reside in their differing evolutionary paths, with *cis*-AT PKS evolution directed by module duplication and domain diversification (Jenke-Kodama and Dittmann 2009) and *trans*-AT PKS evolution guided by horizontal gene transfer between bacteria (Nguyen et al. 2008). As such, *trans*-AT synthases represent a new paradigm in polyketide synthase enzymology.

The unique modular architecture of bacterial PKSs makes these systems ideally suited for re-engineering, an approach that has the potential to deliver non-natural polyketides with improved or novel functionality. Biomolecular re-engineering of PKSs, termed combinatorial biosynthesis, has been a major focus of research activity for over two decades. There have been notable successes in this area, for example modifying the selection and incorporation of variant starter or extender units during the biosynthetic process (Crosby et al. 2012; Koryakina et al. 2013); the successful excision, insertion, or substitution of intact extension modules within or between synthases (Rowe et al. 2001); and the manipulation of tailoring enzymes that catalyze post-PKS site-specific transformations (Tang and McDaniel 2001). However, the ideal of an interchangeable toolkit of synthase components that can be rationally assembled and exchanged to provide a desired chemical or stereochemical output in a final pathway product remains some way from realization. The highly mosaic architectures of *trans*-AT PKSs makes these systems inherently more amenable to re-engineering than their *cis*-AT counterparts. Recent fundamental studies of *trans*-AT PKSs have begun to shed light on the biosynthetic peculiarities of these systems and initial attempts to reprogram their function have been performed. Here we summarize these advances and identify further opportunities for exploiting and directing *trans*-AT PKS enzymology.

Substrate selection and acyl transfer in *trans*-AT PKSs

The defining feature of *trans*-AT PKSs is the absence of integrated *cis*-acting AT domains within each PKS extension module. This essential substrate-loading activity is instead provided in-*trans* through the action of free-standing *trans*-acting ATs encoded within the synthase gene cluster (Cheng et al. 2009). *Trans*-acting ATs act as either stand-alone enzymes, or as embedded functional domains within acylhydrolase (AH) and/or enoyl reductase fusion proteins (Table 1).

In contrast to ATs from *cis*-AT PKSs, and despite recent advances (Musiol et al. 2011; Wong et al. 2011), detailed knowledge of the structures and mechanisms of *trans*-acting ATs remains limited. However, valuable insights into these unusual enzymes are beginning to emerge. *Trans*-ATs incorporate a range of extender units sourced from their respective CoA thioesters. These include most commonly malonyl units, or more unusually acetyl, methylmalonyl, ethylmalonyl, methoxymalonyl, hydroxymalonyl, aminomalonyl, or succinyl units (Menche et al. 2008; Liu et al. 2009b; Cheng et al. 2009; Chan and Thomas 2010; Irschik et al. 2010; Mattheus et al. 2010b; Musiol et al. 2013). Amino acid motifs, identified as being key in dictating substrate selectivity

Table 1 Selected *trans*-AT PKSs and their associated *trans*-acting acyltransferases

Compound	Producing organism	AT	Architecture	Substrate specificity	Evidence for specificity
Albicidin (Royer et al. 2004)	*Xanthomonas albilineans*	AlbXIII	AT	Malonyl-CoA	Sequence analysis
Bacillaene (Calderone et al. 2006); (Bumpus et al. 2008)	*Bacillus subtilis*	PksC	AT	Malonyl-CoA	Experimental
		PksE	AT-ER	Malonyl-CoA	Experimental
Bongkrekic acid (Moebius et al. 2012)	*Burkholderia gladioli*	BonJ	AT	Malonyl-CoA	Sequence analysis
Bryostatins (Lopanik et al. 2010)	*Candidatus endobugula sertula*	BryP	AT-AH	Malonyl-CoA	Experimental
Chivosazols (Perlova et al. 2006)	*Sorangium cellulosum*	ChiA	AT-ER	Malonyl-CoA	Sequence analysis
Corallopyronin A (Erol et al. 2010)	*Corallococcus coralloides*	CorA	AT-ER	Malonyl-CoA	Sequence analysis
Difficidin (Chen et al. 2006)	*Bacillus amyloliquefaciens*	DifA	AT-ER	Malonyl-CoA	Sequence analysis
Disorazols (Wong et al. 2011)	*Sorangium cellulosum*	Dsz/DisD	AT-ER	Malonyl-CoA	Experimental
Elansolids (Teta et al. 2010)	*Chitinophaga pinensis*	ElsA	AT-ER	Malonyl-CoA	Sequence analysis
Etnangien (Menche et al. 2008)	*Sorangium cellulosum*	EtnB	AT	Succinyl-CoA	Sequence analysis
		EtnK	AH-AT	Malonyl-CoA	Sequence analysis
Kalimanticin (Mattheus et al. 2010a)	*Pseudomonas fluorescens*	BatJ	AT	Malonyl-CoA	Sequence analysis
		BatH	AT	Acetyl-CoA	Sequence analysis
Kirromycin (Musiol et al. 2013)	*Streptomyces collinus*	KirCI	AH-AT	Malonyl-CoA	Experimental
		KirCII	AT	Ethylmalonyl-CoA	Experimental
Lankacidins (Arakawa et al. 2005)	*Streptomyces spp.*	LkcD	AT	Malonyl-CoA	Sequence analysis
Leinamycin (Tang et al. 2004; Liu et al. 2009a)	*Streptomyces atroolivaceus*	LnmG	AT-ER	Malonyl-CoA	Experimental
		LnmK	AT-DC	Methylmalonyl-CoA	Experimental
Macrolactins (Schneider et al. 2007)	*Bacillus amyloliquefaciens*	MlnA	AT-ER	Malonyl-CoA	Sequence analysis
iso-Migrastatin (Lim et al. 2009)	*Streptomyces platensis*	MgsB	AH-AT	Malonyl-CoA	Sequence analysis
		MgsH	AT-ER	Malonyl-CoA	Sequence analysis
Mupirocin (El-sayed et al. 2003)	*Pseudomonas fluorescens*	MmpC	AH-AT-ER	Malonyl-CoA	Sequence analysis
Mycosubtillin (Aron et al. 2007)	*Bacillus subtilis*	FenF	AT	Malonyl-CoA	Experimental
Myxovirescins (Calderone et al. 2007)	*Myxococcus spp.*	TaV	AH-AT	Malonyl-CoA	Experimental
Oocydins (Matilla et al. 2012)	*Serratia and Dickeya enterobacteria*	OocV	AT-AH	Malonyl-CoA	Sequence analysis
		OocW	AT	Malonyl-CoA	Sequence analysis
Oxazolomycins (Zhao et al. 2010)	*Streptomyces albus*	OzmC	AT	Methoxymalonyl-CoA	Sequence analysis
		OzmM	AH-AT-ER	Malonyl-CoA	Experimental
Pederin (Jensen et al. 2012)	*Paederus and Paederidus*	PedD	AT	Malonyl-CoA	Experimental

Table 1 continued

Compound	Producing organism	AT	Architecture	Substrate specificity	Evidence for specificity
Rhizopodin (Pistorius and Müller 2012)	*Stigmatella aurantiaca*	RizA	AT	Malonyl-CoA	Experimental
		RizF	AT	Malonyl-CoA	Experimental
Rhizoxins (Partida-Martinez and Hertweck 2007)	*Pseudomonas sp./Rhizopus*	RhiG	AH-AT	Malonyl-CoA	Sequence analysis
Sorangicins (Irschik et al. 2010)	*Sorangium cellulosum*	SorO	AT–AT-ER	AT1 hydroxymalonyl-CoA	Sequence analysis
				AT2 malonyl-CoA	
Spliceostatin A (Zhang et al. 2011)	*Pseudomonas sp.*	Fr9B	AT	Malonyl-CoA	Sequence analysis
		Fr9 J	AT	Malonyl-CoA	
		Fr9O	AT	Malonyl-CoA	Sequence analysis
Thailandamides (Nguyen et al. 2008)	*Burkholderia thailandensis*	TaiC	AH-AT-ER	Malonyl-CoA	Sequence analysis
Thiomarinol (Fukuda et al. 2011)	*Pseudoalteromonas rava*	TmpC	AH-AT-ER	Malonyl-CoA	Sequence analysis
Virginiamycin M (Pulsawat et al. 2007)	*Streptomyces virginiae*	VirI	AT	Malonyl-CoA	Sequence analysis
Zwittermicin A (Kevany et al. 2009)	*Bacillus cereus*	ZmaF	AT	(2*S*)-aminomalonyl-CoA	Sequence analysis

Stand-alone *trans*-acting ATs reclassified as acylhydrolases based on the analysis of Jensen et al. 2012 have been excluded from the table

For completeness these are PksD, bacillaene, BonK, bongkrekic acid, ElsB, the elansolids, PedC, pederin

Abbreviations: *AH* acylhydrolase, *AT* acyltransferase, *DC* decarboxylase, *ER* enoyl reductase

in *cis*-acting ATs can be readily identified in *trans*-acting enzymes thereby allowing substrate selectivity to be reliably predicated using bioinformatic tools.

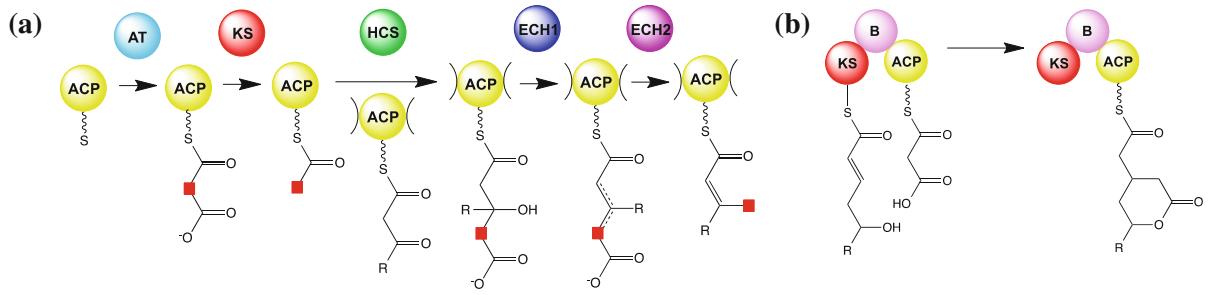
There is a considerable body of published work validating the importance of these motifs in *cis*-acting ATs, including high-resolution structural information, which has permitted the assignment of specific roles to individual amino acids within these regions (Khosla 2009). These observations have proven instrumental in re-engineering AT substrate selectivity. Structural studies of *trans*-acting ATs have demonstrated that these enzymes retain the distinctive 2 sub-domain α/β hydrolase-ferridoxin-like fold of *cis*-acting homologues and that there are determinants of substrate specificity that are common to both families of enzymes (Wong et al. 2011). As such, many of the molecular tools developed for the characterization and manipulation of *cis*-acting ATs would appear to be broadly applicable to *trans*-acting enzymes. The feasibility of altering substrate selectivity through target mutagenesis of *cis*-acting ATs has been elegantly demonstrated. Examples include altering extender unit selection by the AT domain of module 4 (AT4) of the 6-deoxyerythronolide B synthase (DEBS) from methylmalonyl-CoA to malonyl-CoA, which resulted in the production of 6-desmethyl-6-deoxyerythronolide B by the intact synthase (Reeves et al. 2001). Saturation mutagenesis of AT6 of DEBS resulted in the identification of three point mutants with enhanced selectivity for malonyl-CoA over the natural substrate methylmalonyl-CoA. This yielded a modified PKS capable of producing 2-desmethylerythromyin (Sundermann et al. 2013). Data from this study ultimately allowed the design of a variant AT capable of loading an entirely non-natural building block (2-propargylmalonyl) that was readily incorporated into the PKS product yielding 2-propargylerythromycin A. These studies not only illustrate the tractability of modifying AT selectivity through targeted mutagenesis but also demonstrate the downstream tolerance of modular PKSs to accept modified or non-natural extender units.

To date there have been no published examples of *trans*-acting ATs with rationally-altered substrate specificities. Given the robust framework established for the re-engineering of *cis*-acting ATs and the structural and functional similarities between these two families of enzymes, one can be confident that examples of such work will soon emerge. Certainly for detailed in vitro analysis, *trans*-ATs appear to be more attractive targets than *cis*-acting equivalents. They are well-folded, frequently free-standing monomeric enzymes of ~30 kDa in size, and would be considered optimally suited for recombinant expression, purification and subsequent characterization.

The ability of *trans*-acting ATs to acylate multiple ACPs within a single PKS presents intriguing opportunities for exploitation. It is conceivable that by modifying substrate selectivity in a single *trans*-acting AT one could direct extender unit usage within an entire PKS. Further, if substrate promiscuous ATs were employed, capable of selecting and loading a range of acyl substrates, libraries of derivative polyketides could be rapidly assembled in situ, and subsequently isolated and characterized. There are documented examples of substrate promiscuous *cis*-acting ATs with "relaxed" acyl-CoA specificities (Wilson and Moore 2012). PKSs which utilize these transferases are able to biosynthesize multiple variant polyketides in a manner dependent on substrate availability (Mo et al. 2011). This inherent promiscuity has already been exploited to bio-engineer non-natural polyketides (Eustáquio and Moore 2008; Mo et al. 2011) and may well be reproducible in *trans*-acting enzymes through targeted mutagenesis.

There are still significant gaps in our fundamental understanding of *trans*-AT function, most tellingly in how these free-standing enzymes are recruited and orientated at individual extension modules. Clarifying these ambiguities will be key to fully exploiting the potential of *trans*-acting ATs. For example, it is still unknown if *trans*-ATs form stable complexes with PKS extension modules, or if their associations are transient. The specific determinants of AT binding at these sites are currently unknown. Bioinformatic analyses have revealed the presence of remnant fragments of AT domains within *trans*-AT PKS extension modules (Musiol and Weber 2012). These regions of sequence have been termed AT docking domains (ATDs), though there is currently no experimental evidence supporting a role for ATDs in binding and/or positioning ATs during biosynthesis.

Were the details of AT recruitment and binding known, it may be possible to exploit acyltransferase/extension module interactions by directing *trans*-acting ATs with defined substrate selectivities to specific synthase modules, though the ability of *trans*-acting ATs to load acyl substrates onto non-cognate



			AT	KS	ACP	HCS	ECH1	ECH2	ACP
1.	Bacillaene	(Calderone et al. 2006)	PksC	PksF	AcpK	PksG	PksH	PksI	PksL di-ACP
2.	Bongkrekic acid	(Moebius et al. 2012)	BonJ/K	BonF	-	BonG	HonH	BonI	BonD tri-ACP
3.	Bryostatin	(Buchholz et al. 2010)	BryP	BryQ	-	BryR	-	-	-
4.	Corallopyronin	(Erol et al. 2010)	CorA	CorD	CorC	CorE	CorF	CorG	CorK di-ACP
5.	Difficidin	(Tang et al. 2007)	DifA	-	DifC	DifN	-	DifO	DifL di-ACP
6.	Elansolids	(Teta et al. 2010)	ElsA/B	ElsE	ElsD	ElsK	ElsL	ElsM	ElsJ di-ACP
7.	Kalamanticin	(Mattheus et al. 2010b)	BatJ/H	BatB	BatA	BatC	BatD	BatE	Bat2 tri-ACP
8.	Leinamycin	(Tang et al. 2004)	LnmG	-	LnmL	LnmM	LnmF	-	LnmJ di-ACP
9.	Myxovirescin	(Simunovic and Müller 2007)	TaV	TaK	TaB/TaE	TaC/TaF	TaX	TaY	-
10.	Mupirocin	(El-sayed et al. 2003)	Mmplll	MupG	Macp15	MupH	MupJ	MupK	MmpI di-ACP
11.	Oocydin A	(Matilla et al. 2012)	OocV/W	OocF	OocG	OocE	OocD	OocD	OocL di-ACP
12.	Pederin	(Piel et al. 2004)	PedD	PedM	PedN	PedP	PedL	PedI	PedI di-ACP
13.	Spliceostatin A	(Zhang et al. 2011)	Fr9O	Fr9N	Fr9M	Fr9K	Fr9L	-	Fr9H tri-ACP
14.	Thailandamide	(Nguyen et al. 2008)	BTHII1675	1671	1672	1670	1669	1668	1673 di-ACP
15.	Thiomarinol	(Fukuda et al. 2011)	TmpC	TmlG	TacpC	TmlH	TmlJ	TmlE	TmpA tri-ACP
16.	Virginiamycin M	(Pulsawat et al. 2007)	VirI	VirB	-	VirC	VirD	VirE	VirA di-ACP

Fig. 1 Mechanisms of *trans*-AT PKS catalyzed β-branch formation. (**a**) General mechanism of HCS cassette dependent β-branching. (**b**) Mechanism of β-chain extension as typified by the rhizoxin PKS. (**c**) Identities of HCS cassette enzymes and associated ACPs from selected *trans*-AT PKSs. *Dashes* denote pathway components whose identities are yet to be unambiguously established. Abbreviations: *ACP* acyl carrier protein, *AT* acyltransferase, *B* branching domain, *ECH* enoyl hydratase/crotonase, *HCS* hydroxymethylglutaryl *ACP* synthase, *KS* ketosynthase

ACPs has yet to be investigated. If *trans*-acting ATs are capable of loading non-cognate ACPs in heterologous PKSs this could be exploited to produce 'plug-and-play' ATs, freely transferable between systems and hosts. It should be noted that binding interactions between ATs and foreign module components may be impaired due to incompatible protein–protein interaction interfaces. This would likely result in a significant reduction in ACP loading efficiency. Structural and function characterisation of the molecular determinants of these interactions will be crucial for elucidating the specific details of *trans*-AT/extension module complex assembly.

β-Branching in *trans*-AT PKSs

As a consequence of the biosynthetic logic employed by modular PKS, it is often convenient to consider polyketide chains as being comprised of an alternating pattern of carbon atoms occupying either α-or β-positions (Calderone 2008). Alkyl branches at α-carbons are frequently observed in polyketides, and are produced through the action of methyltransferase domains utilizing the substrate *S*-adenosylmethionine (Keatinge-Clay 2012). Branches at β-carbon positions are significantly less frequently observed, due largely to the fact that the electrophilic nature of these atoms requires the action of a nucleophilic alkyl source to permit branch formation. Polyketides produced by *trans*-AT synthases are unusually rich in β-branches, and employ one of two distinct strategies to introduce these substituents (Fig. 1).

The first of these relies on the action of a five protein - (four enzymes, one ACP) hydroxymethylglutaryl-ACP synthase (HCS) cassette, which catalyzes branch formation in a manor analogous to that observed in mevalonate biosynthesis (Calderone

2008; Miziorko 2011). HCS cassette enzymes function in *trans*-assembling branches at designated positions within the growing product chain. Branch formation generally occurs upon distinctive ACP–ACP di-domains, readily identifiable in PKS nucleotide sequences. However, branch formation in some systems has been observed to occur upon tri-domain ACPs (Mattheus et al. 2010a; Fukuda et al. 2011; Moebius et al. 2012).

Amino acid signatures present in ACPs upon which branch formation occurs have been identified (Haines et al. 2013). These appear to direct HCS-cassette enzyme recruitment and binding, affording a level of positional control over branch placement. One attractive hypothesis is that ACPs upon which branches are assembled may be relocated to alternative modules within their PKS of origin, or along with their partner HCS-cassette proteins into heterologous systems, to permit targeted branch formation at designated sites within the polyketide chain. Given that alkyl branches are further addressable by synthetic or biosynthetic methods, these new chemical handles could act as precursors for the introduction of a range of functional groups. Such modifications have been observed previously in PKSs, resulting for example in the formation of cyclopropane or vinyl chloride substituents (Edwards et al. 2004; Gu et al. 2009). When repositioning branching ACPs within or between systems the down-stream processivity of any branched products must be considered. Ketosynthase domains that catalyze chain extension of branched products possess distinctive active site architectures that permit β-branches to be accommodated (Jenner et al. 2013). It is recommended that if relocation of branching ACPs is attempted ACP-KS domain fusions be used in preference to isolated ACPs.

A second recently identified strategy for β-branching in *trans*-AT PKSs relies on the action of a unique branching (B) domain containing extension module, which catalyzes branch formation via a Michael-type acetyl addition (Bretschneider et al. 2013). This distinctive β-chain extension chemistry further diversifies the palette of *trans*-AT PKS enzymology and presents intriguing opportunities for synthase re-engineering. Unlike HCS-cassette-dependent branching the functional components required for branch formation and polyketide chain extension are housed within a single synthase module. It is conceivable therefore that this intact unit could be relocated within or between PKSs to facilitate branch incorporation. In instances where the introduction of a single β-branch in a polyketide product is required this may be a more attractive strategy than using a HCS cassette based approach. In contrast, where the insertion of multiple branches at different positions within a product is needed, exploiting HCS cassette branching offers considerable benefits, as branch formation at multiple sites can be directed from a single pool of *trans*-acting enzymes. The success of either approach will be highly dependent on the correct placement of relocated domains or modules at selected sites within the target synthase. The authors suggest that where possible high resolution structural information be used to guide the placement process. This will ensure that inter-domain linkers and protein–protein interaction interfaces can be clearly defined and if necessary optimized.

Hybrid *trans*-AT synthases

Polyketide synthases are not the only example of modular assembly-line like megaenzymes. The sequential chain extension chemistry employed in PKSs is also seen in non-ribosomal peptide synthetases (NRPSs) that synthesize polymeric peptidic natural products. There are numerous enzymological features common to both PKSs and NRPSs. In addition to sharing a modular architecture, both use carrier proteins as sites of attachment for extender unit substrates and the growing product chain, with peptidyl carrier proteins (PCPs) performing this role in NRPSs. Both rely on the action of phosphopantetheinyl transferases to post-translationally modify carrier proteins permitting extender unit and product chain attachment. NRPS modules house an adenylation domain (A), akin to a PKS AT, which selects and loads proteinogenic or non-proteinogenic amino acids onto neighboring PCPs. Chain extension is catalyzed by condensation domains (C) that fuse aligned peptidyl and amino acyl thioesters, reminiscent of KS domains in PKS modules. Given these striking similarities, it is unsurprising that hybrid synthases composed of PKS and NRPS components have been identified. *Trans*-AT PKSs, with their highly mosaic architectures, are frequently found to house NRPS modules and many of the *trans*-AT synthases characterized to date contain these components. By marrying

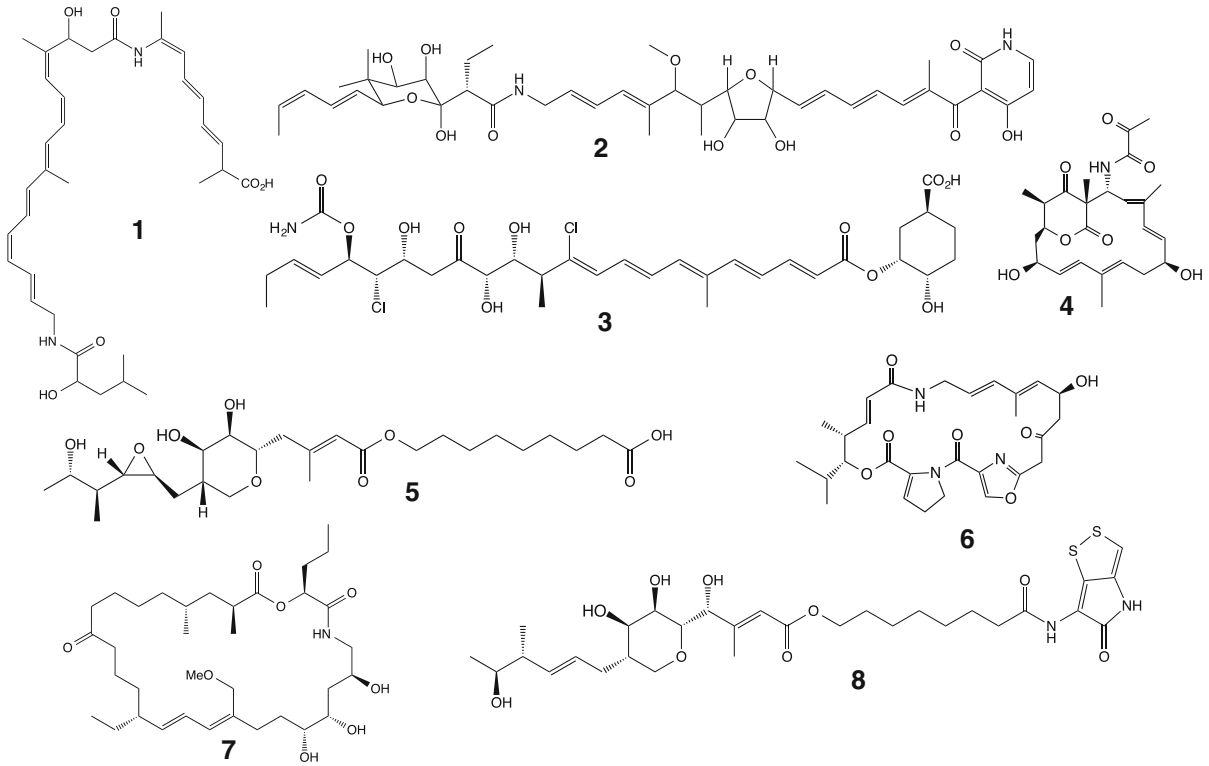


Fig. 2 Examples of natural products biosynthesized by hybrid *trans*-AT PKSs. (*1*) bacillaene, *trans*-AT PKS/NRPS; (*2*) kirromycin, *cis*/*trans*-AT PKS/NRPS; (*3*) enacyloxin IIa, *cis*/*trans*-AT PKS/NRPS; (*4*) lankacidin C, *trans*-AT PKS/NRPS; (*5*) mupirocin, *trans*-AT PKS/FAS; (*6*) virginiamycin M, *trans*-AT PKS/NRPS; (*7*) myxovirescin A, *trans*-AT PKS/NRPS; (*8*) thiomarinol A, *trans*-AT PKS/FAS/NRPS

polyketide and peptide chemistries, hybrid systems posses the capacity to assemble products of diverse chemical structure and biological function (Fig. 2). For this reason they represent highly attractive targets for re-engineering.

A powerful approach for re-engineering NRPS modules in hybrid systems is through the manipulation of A domain specificity, such that non-native amino acids may be incorporated into product scaffolds. This may be achieved by domain substitution or targeted mutagenesis (Evans et al. 2011; Thirlway et al. 2012). The former approach has been used successfully, though is often associated with decreased product yield, a likely consequence of domain interface incompatibility (Fischbach et al. 2007; Williams 2013). The latter has been made tractable by the establishment of an A domain 'code' permitting the prediction of substrate specificity and is based on inspection of A domain, amino acid sequence (Marahiel et al. 1999; Challis et al. 2000). Through the substitution of key active site residues A domain substrate selectivity can be rationally altered to facilitate the incorporation of a range of amino acids (Fischbach et al. 2007). In addition, NRPS A domains with promiscuous substrate selectivities have been identified. Harnessing the potential of these domains could result in the production of libraries of derivative compounds from a single synthase, expanding yet further the biosynthetic potential of hybrid systems. Convergent pathways marrying *trans*-AT PKSs and NRPSs have also been observed, typified by the thiomarinol synthase. In this system, pseudomonic acids similar to the *trans*-AT PKS polyketide mupirocin, itself a *trans*-AT PKS/fatty acid hybrid, are fused to a holomycin like pyrrothine moiety (Fukuda et al. 2011). The enzyme, TmlU, encoded within the thiomarinol gene cluster has been proposed to join the two components in a manner reminiscent of that observed for SimL and NovL in the simocylinone and novobiocin pathways respectively (Steffensky et al. 2000; Luft et al. 2005). Such amide ligase activity appears highly exploitable, and could be used to fuse

polyketide and peptide fragments without the need to relocate or mutagenize NRPS domains within synthases. This would minimize associated deleterious effects. The tolerance of these enzymes to acyl and peptide substrates is yet to be comprehensively assessed, though if limited could be expanded by rational active site redesign.

Despite their different evolutionary origins, both *cis*-AT and *trans*-AT PKSs employ the same biosynthetic logic to assemble polyketide products. There appears, therefore, no obvious impediment to *cis*-AT and *trans*-AT extension modules coexisting within a single synthase. It is perhaps surprising, therefore, that it is only recently that such hybrid *cis*-AT/*trans*-AT PKSs have been identified. To date five such systems have been reported of which two, the kirromycin and enacyloxin PKSs, have been subjected to detailed characterization (Weber et al. 2008; Mahenthiralingam et al. 2011; Behnken and Hertweck 2012).

Both the kirromycin and enacyloxin gene clusters contain ORFs encoding *cis*-AT and *trans*-AT PKS modules and though based on gene cluster analysis, the two module types are not co-located within a single polypeptide chain but are housed separately as embedded components within different proteins. Both systems contain *cis*- and *trans*-ATs with predicted specificity for malonyl-CoA highlighting degeneracy in substrate usage and suggesting that these systems are likely to of arisen through the convergence of *cis*-AT and *trans*-AT PKSs. These natural systems illustrate the capacity of *cis*- and *trans*-AT components to be accommodated together, further expanding the potential for diversifying product chemistry through the re-engineering of *trans*-AT systems, specifically through the incorporation of *cis*-AT elements. Many of the tools developed for re-engineering *cis*-AT PKSs would consequently be directly applicable to the manipulation of *cis*/*trans*-AT hybrids. As more sequence data emerges from genome mining studies we predict that *cis*/*trans*-AT systems will become an increasingly common sight, and as a consequence new biosynthetic capability and routes to pathway re-engineering will emerge.

Conclusion

The speed and reliability of modern genome sequencing along with the increasing volume of detailed structural and functional data that is now available is providing biomolecular engineers with a strong platform for manipulating modular PKS function. *Trans*-AT PKS re-engineering shows considerable promise, with these systems exhibiting a novelty and diversity of function far in excess of *cis*-AT equivalents. Significant further fundamental insight into *trans*-AT synthases will be required before these systems can be rationally and reproducibly reengineered, although examples of this work are beginning to emerge and there is evidence to suggest that at least a subset of the molecular, genetic and analytical tools developed for manipulating *cis*-AT PKSs will be broadly applicable to *trans*-AT systems. As an increasing number of *trans*-AT PKSs and hybrids thereof are identified and characterised, so to increases the scope and potential for synthase re-engineering. Undoubtedly the resulting marriage of pathway discovery and combinatorial biosynthesis, expedited by modern synthetic biology methods, will provide access to an unprecedented diversity of new natural products of clinical and agrochemical significance.

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