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On-line enzymatic tailoring of polyketides and peptides in thiotemplate systems

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Non-ribosomal peptide synthetases (NRPS) and type I polyketide synthases (PKS) are versatile thiotemplate systems for the programmed assembly of biosynthetic building blocks. Typically, the post-PKS/NRPS enzymes tailor the resulting chains to yield the bioactive natural product scaffolds. However, more and more examples have surfaced showing that important structural modifications take place while the intermediates are still bound to the assembly line. A growing number of enzymatic domains and trans-acting enzymes as well as their recruiting areas in the modules have been identified and characterized. In addition to the widespread on-line alkylations, hydroxylations and heterocyclizations into oxazole/thiazole residues, on-line modifications lead to a variety of ring systems such as cycloethers, lactones, lactams, glutarimides, cyclopropanes, decalins and cyclic biaryls.

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Introduction

Thiotemplates are fascinating microbial multienzyme systems that produce a wealth of polyketide and peptide natural products, many of which are medicinally important as antibiotics, anticancer drugs and immunosuppressants. These versatile molecular assembly lines have a modular architecture with specialized domains for substrate loading, assembly, modification and chain release. Depending on the type of building blocks and mechanisms employed for the chain assembly, thiotemplates fall into two groups: type I polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS), yet there are many hybrids that generate mixed structures [1–4].

By contrast to biosynthetic pathways where several free-standing enzymes catalyze the stepwise transformation of free-floating, non-covalently bound substrates, in thiotemplates the growing polyketide or peptide chains are tethered to the phosphopantetheinyl arms of acyl-carrier protein (ACP) or peptidyl-carrier protein (PCP) domains until full maturation of the polyketide or peptide backbones. Thus, the thiotemplate systems prevents diffusion of the substrates and ensures spatial and temporal control over the many fine-tuned biochemical processes, defining the choice of building blocks, order of incorporation, degree of processing and the size of the chains. In addition, the terminal modules or domains determine the fate of the product (Figure 1). Typically, thioesterase (TE) domains liberate the products by either simple hydrolysis or (macro)cyclizations [5,6•], including dimerizations to create macrodiolides [7,8]. Alternatively, reductive chain release, aminolysis as well as various more sophisticated off-loading mechanisms are known, which may lead to tetronates and several other ring systems (Figure 1b) [9,10•]. Despite these variations governed by chain-termination processes, according to text-book knowledge thiotemplates are mainly in charge of assembling the basic peptide and polyketide scaffolds, whereas so-called post-PKS and post-NRPS enzymes are responsible for tailoring reactions such as oxygenations, glycosylations and cyclizations, which are often crucial for the biological activities of the final products. As the names post-PKS and post-NRPS imply, chain assembly and tailoring reactions would be time-resolved events. However, with the growing number of studied biosynthetic pathways involving thiotemplates, more and more examples surfaced where *trans*-acting enzymes or novel PKS/NRPS domains catalyze peptide/polyketide transformations during chain assembly.

Common on-line modifications (alkylations, hydroxylations, and amino acid heterocyclizations)

In addition to the canonical NRPS and PKS domains for β-keto processing and amino acid epimerization, domains for on-line hydroxylation and methyl transfer are widespread. In particular, integral SAM-dependent methyltransferase (MT) domains for α-methylation are frequently found in *trans*-AT PKSs, a subgroup of bacterial modular PKSs in which the malonyl units are loaded by a *trans*-acting acyl transferases (AT) [11]. For the same type of PKSs, another on-line alkylation mechanism is prevalent that leads to terpenoid-like alkyl branches at the β-carbon relative to the thioester. A set of enzymes encoded by the hydroxymethylglutaryl-CoA synthase

(HCS) gene cassette is in charge of mediating the β-branching reactions in *trans* [12]. Specialized motifs in the PKS warrant the formation of the HCS-ACP complex in the correct module [13].

Perhaps the best-known on-line cyclization reactions are the cyclodehydrations of serine and cysteine residues of peptides into oxazoline and thiazoline rings, respectively, which are often found in siderophores (Figure 1c). For this common modification, NRPS modules harbor cyclization (CY) domains, which may be paired with oxygenase (OXY) domains for the formation of oxazole and thiazole heterocycles [6•]. Such backbone cyclizations are often important for the overall architecture of the natural products and rigidify the scaffold. The following sections highlight recent discoveries of on-line tailoring reactions with emphasis on cyclizations (Figure 1d).

Cycloether formation during polyketide biosynthesis

Cycloethers are often important moieties that confer biological activity to polyketide metabolites. Prominent examples are polyether ionophores like monensin and lasolocid. Most often, the oxacycles are incorporated by epoxidation followed by intramolecular nucleophilic epoxide ring opening catalyzed by oxygenases and epoxide hydrolases/cyclases [14–16]. Tetrahydrofuran and tetrahydropyran rings may also be formed by vinylogous addition reactions and cytochrome-mediated C–O-bond formation, yet until recently all of these reactions were known as typical post-PKS modifications.

Recently, specific PKS domains have been discovered that mediate polyketide heterocyclization during chain assembly. One such example is the dehydratase (DH) domain involved in the biosynthesis of a potent haterumalide, oocydin A (**5**) produced by plant-associated enterobacteria of the *Serratia* and *Dickeya* genera. One of the two DH domains mediates the attack of a hydroxyl group to the β-postion of an α, β-unsaturated thioester in a Michael addition reaction, resulting in a furan ring (Figure 2a) [17]. A similar reaction is shown to be catalyzed by a pyran synthase-like (PS) domain in the biosynthesis of phormidolide A (**6**), a brominated macrocyclic polyketide isolated from a marine cyanobacterium (*Leptolyngbya* sp.) (Figure 2b) [18]. In both cases, the catalytic domains are part of a type I *trans*-AT PKS. The PS domain is also identified in the biosynthesis of pederin (**7**), an antimitotic agent produced by a symbiont (*Pseudomonas* sp.) of rove beetles (*Paederus* spp.) [19]. The catalytic domain, which is part of a type I *trans*-AT PKS, exhibits moderate homology to DH domains with mutated active site residues. The PS domain was shown to mediate the oxa-conjugate addition in a stereocontrolled manner. As a stand-alone enzyme the PS domain is capable of forming 5-membered and 6-membered cycloethers, thus representing a versatile biocatalyst (Figure 2c) [20]. Notably, till date no PS-like domains have been identified in *cis*-AT PKS systems. However, a functionally related DH domain with both dehydratase and cyclase activities plays a key role in the biosynthesis of ambruticin (**8**), an antifungal agent produced by the myxobacterium *Sorangium cellulosum*. Analysis of the ambruticin modular (*cis*-AT) PKS and *in vitro* assays using synthetic substrate analogs showed that the DH3 domain catalyzes a dehydration-cyclization cascade to yield the pyran ring (Figure 2d) [21].

A tetrahydropyran ring is also found in the structure of salinomycin (**9**), a polyether ionophore from *Streptomyces albus*, yet in contrast to the above-mentioned pathways, no 'PS' or 'DH'-like domains can be found in the modular salinomycin PKS. It was shown that pyran ring formation requires a novel cyclase, SalBIII, which is similar to the MonB-type of epoxide hydrolases. By contrast to the conventional epoxide ring opening reactions typically mediated by such enzymes, SalBIII catalyzes an E1cb-like β-elimination reaction (Figure 2e) [22]. It was proposed that SalBIII acts in trans to transform the PKS-bound intermediate into the oxacycle. The crystal structure of the enzyme has been solved, which revealed two aspartate residues (Asp38 and Asp104) in the active site, which are implicated in the elimination-addition reaction. On the basis of gene cluster analyses it is conceivable that an integral Cyc11 domain of the indanomycin PKS mediates pyran ring formation by a similar mechanism [23]. Taken together, the PS and DH domains of the pederin and ambruticin PKSs and the novel *trans*-acting SalBIII cyclase broaden the range of online polyketide heterocyclizations.

Polyketide branching with lactonization, glutarimide formation and lactamization

In addition to cycloether biosynthesis, on-line Michael additions are also involved in the formation of various other pharmacophoric heterocycles such as δ-lactone and glutarimide moieties that branch off the polyketide backbone. Contrary to O-heterocyclization, these additional ring systems are installed by the vinylogous attack of an α,β-unsaturated thioester intermediate by a C-nucleophile, produced by decarboxylation of the ACP-bound malonyl extender unit, followed by cyclization. First insights into this unusual polyketide branching mechanism were gleaned from analysis of the modular *trans*-AT PKS for the antimitotic phytotoxin rhizoxin (**10**) (Figure 3a) that is produced in symbiosis of the fungus *Rhizopus microsporus* and its endobacterium *Burkholderia rhizoxinica* [24,25] Elucidation of the pathway intermediates and the assembly line program [26] suggested that a designated branching module (KS (Ketosynthase)-B (Branching)-ACP) plays a key role in chain branching. By the successful *in vitro* reconstitution of the module it was found that this set of domains is sufficient to catalyze the entire branching/cyclization sequence. According to results obtained from stable-isotope labeling experiments and trapping