Aureothin Polyketide Synthase

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Multifactorial Control of Iteration Events in a Modular Polyketide Assembly Line**

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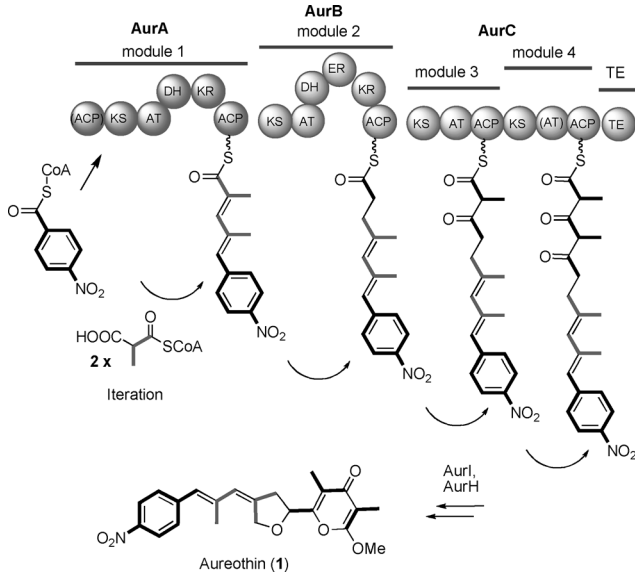
Complex bacterial polyketides such as polyenes, macrolides, and polyethers are a rich source of clinically used therapeutics. Despite their highly diverse structures, these valuable compounds are assembled from simple acyl and malonyl building blocks in an enzymatic assembly line.[1] The polyketide synthases (PKSs) in charge of selecting, fusing, and processing the building blocks are organized into modules that consist of individual catalytic domains. Typically, there is a unidirectional progress of chain elongation, where each domain is only used once in the biosynthesis of one polyketide molecule. Thus, for most synthases there is a strict colinearity between the number and architecture of the modules and the number of elongations and degree of β-keto processing.[2] The beauty of this rule of colinearity is that it is possible to deduce the structure of a polyketide metabolite from the organization of the PKSs and vice versa. In addition, the rule of colinearity has proven extremely valuable for rationally engineering PKSs[3–6] and to predict structures of cryptic natural products from genome sequences by genome mining.[7,8] However, there are more and more deviations from this textbook knowledge; various modular PKS single domains or entire modules are skipped and individual modules may be used more than once.[2] Notably, in contrast to an erratic processing of the PKS, these events are programmed and apparently have developed during the evolution of the assembly lines. The first examples for the programmed iterative use of a type I PKS module were observed in the stigmatellin,[9] aureothin[10] (each two iterations), and borellidin[11] (three iterations) pathways. Module fusions of the aureothin and borrelidin PKSs proved that individual modules are responsible for the iterative processes.[11,12] Recently it was shown that iteration takes place by retrotransfer of the intermediate from one PKS strand to the opposite PKS strand,[13] and that a ratchet mechanism of KS–ACP interactions precludes iterative use of any single module in colinear systems.[14] Despite the growing number of non-colinear PKSs,[1] to date the mechanisms that control the iteration processes in non-colinear modular PKSs have remained obscure.[15] A possible scenario could be that iteration is an inherent feature of the module as, for example, in fatty acid synthases or fungal PKSs. Alternatively, the module may be "forced" to iterate because the downstream module only accepts an intermediate with a particular chain length. A combination of both is possible, too. Finally, the kinetics of iteration and de novo loading of the ketosynthase needed to be fine-tuned to allow for an unobstructed metabolite flux. Herein, we report the first insights into the control of iteration in a modular PKS and reveal that the interplay of multiple components is essential to control the exact number of elongation cycles.

The aureothin PKS from *Streptomyces thioluteus* (Scheme 1) represents a favorable model system to investigate the iteration process because the size of the gene cluster allows engineering and expression studies in heterologous hosts (such as *Streptomyces albus*). As no aureothin homologues with shortened or extended polyene stretches are produced naturally, the iteration event in the biosynthesis of aureothin is tightly controlled. Furthermore, a particular feature of the *aur* PKS is that the iterating module is, at the



Scheme 1. Model of aureothin (**1**) biosynthesis. Non-colinear assembly by a modular PKS. AT = acyltransferase, DH = dehydratase, ER = enoyl reductase, KR = ketoreductase, KS = ketosynthase, TE = thioesterase.

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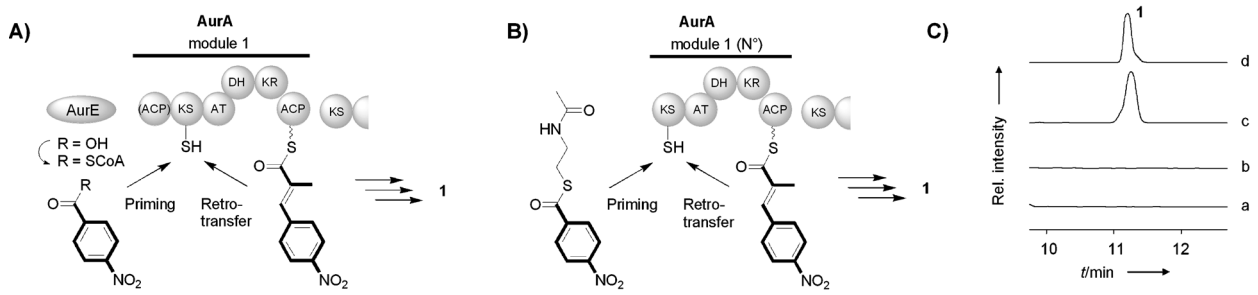


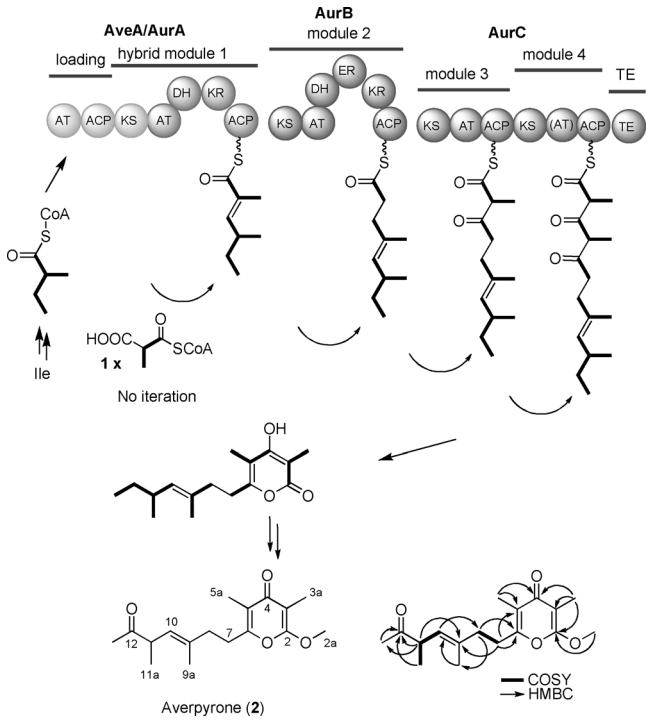
Figure 1. Investigation of the priming of the iterative module (AurA). A) The ligase AurE solely plays a role in activating the *p*-nitrobenzoate (PNBA) starter unit. For iteration, KS1 must control the rates of priming and retrotransfer of the diketide. B) Aureothin biosynthesis is functional in the absence of the N-terminus of AurA, and a Δ*aurE* mutant can be complemented by a synthetic SNAC derivative. C) Metabolic profiles of a) *S. lividans* ZX1:pNT38 (Δ*aurE* mutant), b) Δ*aurE* mutant supplemented with PNBA, c) Δ*aurE* mutant supplemented with PNBA-SNAC, d) Δ*aurE* mutant complemented with *aurE* (expression plasmid).

same time, the chain initiating PKS module that selects the *p*-nitrobenzoate starter unit.[16,17] A prerequisite for the iterative use of the first module is that the thiol group of the KS1 domain remains vacant. Otherwise, an occupied active site of the KS domain loaded with the starter unit would hamper the second elongation step. In consequence, the reconveyance of the diketide intermediate must proceed faster than the priming of the first module (Figure 1). For this reason, we first investigated the loading of the *aur* PKS with the starter unit. Because the *aur* PKS lacks a designated loading module, we focused on the *N*-terminal part of the first module AurA and the acyl-CoA ligase AurE, an enzyme that often assists in loading a starter unit onto the PKS assembly line.[18] A mutant lacking the gene for the acyl-CoA ligase was created by excision of *aurE* from the *aur* gene cluster. Interestingly, aureothin production was completely abolished in this Δ*aurE* mutant. Genetic complementation fully restored aureothin production, thus excluding any polar effects of the mutant. Notably, we could also chemically restore aureothin biosynthesis in the *aurE* mutant when adding the CoA surrogate, the *N*-acetyl cysteamine thioester (SNAC) of *p*-nitro benzoic acid.[19] This result clearly showed that AurE is solely involved in starter unit activation, but does not participate in PKS priming. To rule out the involvement of the *N*-terminal part of AurA, we also tested the truncated mutant and found that the *N*-terminus of AurA is not essential for priming the aureothin PKS. These findings implied that the KS alone has full control over priming and chain elongation events.

Next, we investigated the impact of the KS domains on the iteration process. To exchange the KS1, two single restriction sites flanking the gene region coding for the AT domain were introduced in *aurA*. Using this construct, the gene region for the KS1 was exchanged by the gene region coding for the loading module and the KS1 domain of the avermectin synthase (AVES1).[20–22] The newly generated hybrid module was introduced into a mutant lacking wild-type *aurA*. HPLC-MS analysis of the metabolic profile of the recombinant strain revealed the formation of a new compound.

The metabolite of the *ave/aur* hybrid PKS, named averpyrone, was isolated from large-scale fermentation broth, and its structure was fully elucidated by HRMS, IR, and both 1D and 2D NMR spectroscopy. For carbons 2–10, the NMR data for averpyrone were quite similar to the ones for deoxyaureothin. C11 (δ 46.0) has a distinct chemical shift compared to C11 (δ 139.3) in deoxyaureothin,[23] thus indicating a carbonyl next to this position. This is supported by the coupling of H11 (δ 3.34) as a quartet of doublets with H11a and H10. No coupling with other protons could be observed in the ^{1}H NMR spectrum of H13 (δ 2.05), which suggests a connection to a quaternary carbon. The chemical shift of C12 (δ 203.1) is shifted to lower field. The HMBC coupling of H11, H11a, and H13 with C12, along with the deduced molecular composition, established the keto group in position C12. Whereas averpyrone has been regioselectively methylated by AurI,[24] it lacks the oxygen heterocycle of aureothin. Apparently, **2** is not the correct substrate of the multifunctional P-450 monooxygenase AurH, which usually catalyzes hydroxylation–heterocyclization at C7/C9a.[23,25] As it has been shown that AurH may install carbonyl groups into non-natural substrates,[26–28] it is possible that it also introduced the oxo group at C12 (Scheme 2).

Averpyrone features a 2-methylbutyryl side chain in lieu of the *p*-nitrophenyl residue. To unequivocally prove the origin of this moiety, $^{13}C_6$-L-isoleucine was added to the engineered hybrid PKS strain. MS analyses revealed a mass shift of 5 Da that clearly showed the intact incorporation of a 2-methylbutyrate unit that originates from L-isoleucine by means of the branched chain alpha-keto acid dehydrogenase complex (BCKD).[18] The structure of **2** and the labeling experiments imply that 2-methylbutyl-CoA, which is loaded onto the *ave/aur* hybrid PKS, undergoes only four rounds of elongation. In the hybrid synthase, the number of modules and the number of elongation steps are in perfect agreement with the rule of colinearity. Clearly the first module had lost its ability to catalyze two rounds of elongations. Consequently, the genuine KS1 domain of AurA plays a crucial role in the iteration event. The successful production of averpyrone has yet another implication. The size of its backbone and the methyl branching pattern of the biosynthetic intermediate produced by the hybrid module resemble the intermediate produced by AurA. The (*E*)-2-methylpent-2-enoyl moiety is accepted as a substrate by KS2 and all other downstream PKS domains. We thus tested a range of alternative starter units that are usually loaded onto the *ave* PKS, however, only the branched building block was incorporated (data not shown). It appears that the KS has specificity for substrates with



Scheme 2. Loss of iteration in an engineered variant of AurA. Structure of the hybrid metabolite averpyrone, and selected COSY and HMBC correlations.

a particular substitution pattern in analogy to what has been observed for KS from *trans*-AT systems.[29,30] To further evaluate the potential gatekeeper function of the KS2, we prepared SNAC analogues of polypropionate intermediates with varying chain lengths and administered these probes to the Δ*aurA* mutant.[31] Unfortunately, in all cases the synthetic mimics were degraded. We also heterologously produced KS domains for in vitro studies, but the soluble proteins were unfortunately nonfunctional as standalone entities. Finally, we chose to analyze the pathway intermediates in more detail in vivo. Specifically, we compared intermediates produced by AurA alone and those formed within the context of the entire assembly line.

To promote a premature release of biosynthetic intermediates, we deleted the C terminal thioesterase domain of the *aur* PKS.[32–34] Initially, the mutant lacking the off-loading machinery was cultured at 28 °C for 5 days, after which no production of any biosynthetic intermediate was observed. To rule out degradation of the biosynthetic intermediates, we systematically varied the cultivation times. By LC-HRMS monitoring of the 12–24 h old main culture, we were able to detect trace amounts of metabolites. The deduced molecular composition corresponded well with the expected biosynthetic intermediates of modules 1 and 2 (Scheme 1). Only the

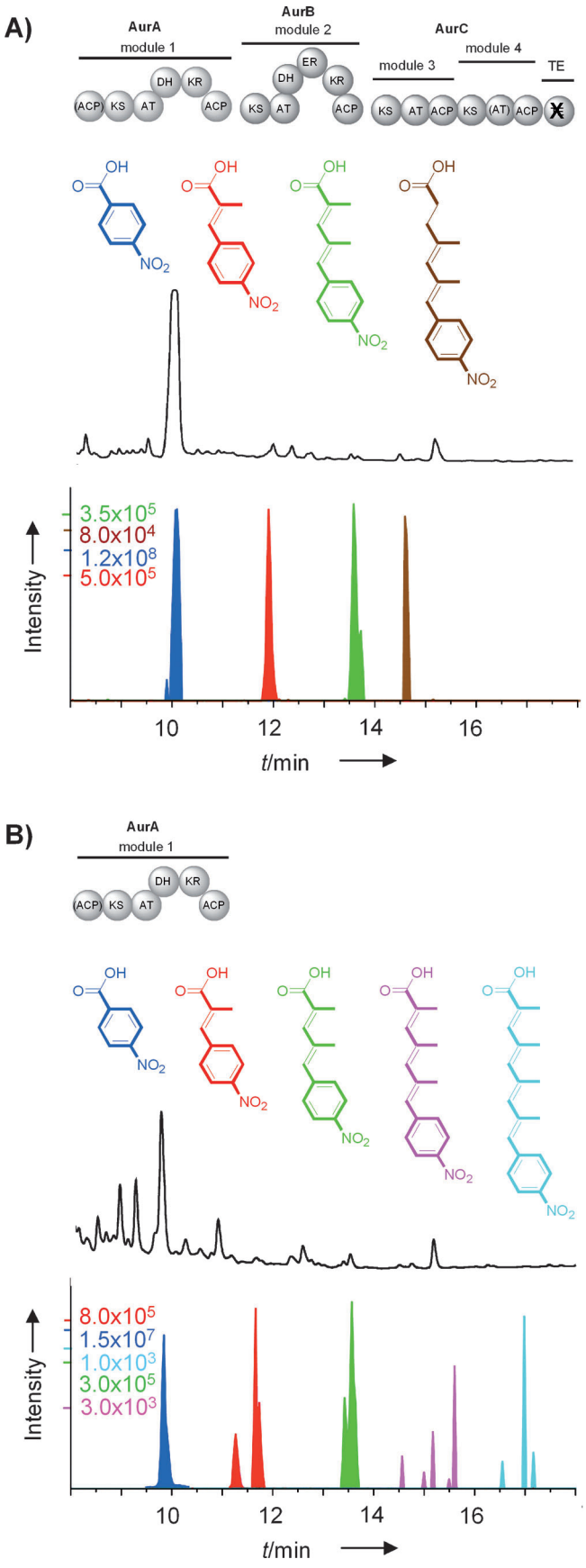


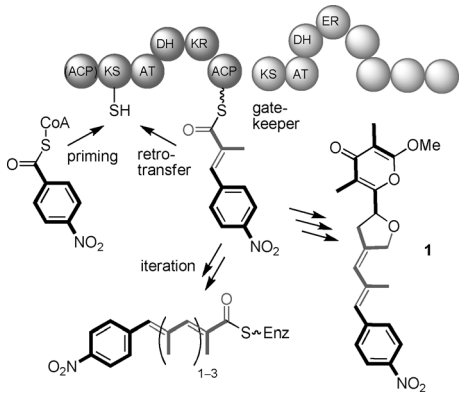
Figure 2. Dissection of the *aur* PKS and analysis of polyketide intermediates by LC-HRMS; extracted ion masses are color coded to match the synthetic references. A) Prematurely off-loaded intermediates detected from a mutant lacking the thioesterase (TE) domain. B) Multiple products of the heterologously produced, freestanding iterative module (AurA); multiple peaks refer to *E*/*Z* isomers.

predicted intermediate of module three was not observed; this is likely due to the instability of the β-keto acid. As isolation of these products was not feasible owing to the low quantities produced, we synthesized authentic references of the postulated aureothin intermediates (see the Supporting Information) and compared their retention times with the observed metabolites. All proposed intermediates of AurA and AurB could be clearly identified. However, no metabolites with shorter or longer backbones were produced.

Next, we investigated the polyketides generated by the iteratively operating module AurA alone. For this purpose, *aurA* and *aurE* were cloned into an expression vector and introduced into *S. lividans* ZX1. The resulting strain was cultured with supplementation of PNBA, and the metabolic profile was again monitored by LC-HRMS. As before, we detected molecular masses that correspond to the di- and triketide intermediates. Surprisingly, we also observed masses that pointed to polyketides resulting from three and four rounds of elongations (Figure 2). To rigorously determine the identity of these compounds, we also prepared authentic references for these metabolites and compared their masses and retention times with the observed products by LC-HRMS. Multiple peaks indicated *E*/*Z*-isomerizations, which readily take place when exposing polyunsaturated polypropionates to daylight.[35]

The unexpected finding of polyketide metabolites of AurA that have undergone up to four chain elongations indicates that the first module possesses an intrinsic ability to work iteratively and that the repetitive usage of the first module is not a result of its context within a multimodular PKS. Furthermore, these results demonstrate that the first module is, in principle, able to perform more rounds of iteration than what is observed within the context of the entire assembly line. Even though the triene and tetraene products are formed only in minute amounts, it should be highlighted that they cannot be observed in the intact assembly line. Thus, the KS2 has specificity for a defined chain length and readily processes the correct diene intermediate, thus preventing further elongation rounds. Taken together, the KS1 clearly mediates iteration, but the downstream module (KS2) serves as a gatekeeper to control the correct assembly of the aureothin polyketide backbone (Scheme 3).

In summary, we have gained first insights into the factors governing the iteration processes in a non-colinear PKS. Through gene deletion and complementation with a synthetic surrogate, we found that the KS1 of the iterating module is exclusively in charge of priming the PKS. This is a prerequisite to permit a vacant position for the retrotransfer of the diketide. The model of kinetic control is fully in line with the observed complete loss of iteration in an engineered AurA variant harboring the designated loading module of the avermectin PKS. Finally, through deletion of the TE domain and the use of synthetic reference compounds, we succeeded in detecting the polyketide intermediates of iterative and non-iterative *aur* PKS modules. Whereas this experiment confirmed a high fidelity of the programmed events, the iterative module alone produces polyketides with varying lengths that correspond to one to four rounds of elongations. Thus, we



Scheme 3. Model for the multifactorial control of non-colinear chain elongations in aureothin biosynthesis.

provide strong experimental support for a scenario where multiple factors are responsible for the rare iteration process. It is clearly an inherent quality of the KS1 to mediate priming, chain propagation, and intermediate retrotransfer, but the downstream KS2 functions as a gatekeeper that excludes the propagation of intermediates that are either too short or too long. Beyond new insights into the hidden program of a noncanonical multimodular assembly line, our findings also provide valuable information for the rational design of complex polyketides by pathway engineering.

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Communications

Aureothin Polyketide Synthase

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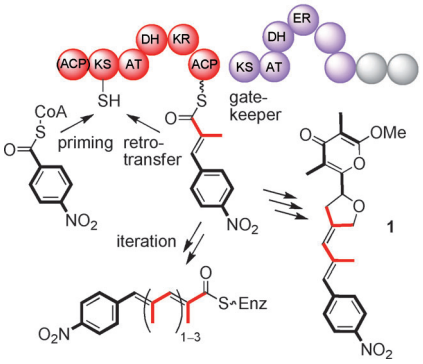
Multifactorial Control of Iteration Events in a Modular Polyketide Assembly Line



Freedom and control: First insights into the rare programmed iteration of an individual polyketide synthase (PKS) module were obtained from the analysis and mutation of aureothin (**1**) synthase. The first ketosynthase (KS) domain primes the PKS, allowing intermediate retrotransfer. Addition of a designated loading module results in a complete loss of iteration. The downstream KS functions as a gatekeeper for correct chain length.