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Interchenar Retrotransfer of Aureothin Intermediates in an Iterative Polyketide Synthase Module

Benjamin Busch, Nico Ueberschaar, Yuki Sugimoto, and Christian Hertweck*

Leibniz Institute for Natural Product Research and Infection Biology, HKI, Beutenbergstrasse 11a, 07745 Jena, Germany, and Friedrich Schiller University, Jena, Germany

Supporting Information

ABSTRACT: The course of the enigmatic iterative use of a polyketide synthase module was deduced from targeted domain inactivation in the aureothin assembly line. Mutational analyses revealed that the N-terminus of AurA is not involved in the iteration process, ruling out an ACP−ACP shuttle. Furthermore, an AurA(KS°, ACP°)−AurA(AT^0) heterodimer proved to be nonfunctional, whereas aureothin production was restored in a Δ*aurA* mutant complemented with AurA(KS°)−AurA(ACP°). This finding supports a model according to which the ACP-bound polyketide intermediate is transferred back to the KS domain on the opposite PKS strand.

Modular polyketide synthases are fascinating multifunctional biocatalysts involved in the biosynthesis of many pharmacologically relevant natural products. These giant enzymes form a molecular processing line to assemble complex polyketide metabolites from simple acyl and malonyl units.[1−3] In general, a distinct set of catalytic domains forming one module is utilized for one elongation and processing cycle before the tethered intermediate is transferred to the downstream module. The number of active domains in the PKS typically corresponds with the number of required biotransformation steps, which allows for the prediction of the basic polyketide skeleton from the PKS architecture and vice versa. However, there is a growing number of exceptions to this rule of colinearity.[4] Beside the skipping of individual domains or entire modules, also the irregular reuse of a domain or one module, referred to as 'stuttering', has been reported.[5] However, only few modular systems were discovered where the iterative usage of individual PKS modules is a programmed event in the biosynthesis.[4] First, direct evidence for the iterative usage of one module was provided by the functional analyses of the borrelidin (*bor*)[6] and aureothin (*aur*)[7,8] synthases (Figure 1A), which have been shown to catalyze three and two rounds of Claisen condensations, respectively. Other rare examples of iterative modules are known from stigmatellin,[9] neoaureothin,[10] neocarzilin,[11] lankacidin,[12] and DKxanthene[13] PKSs, as well engineered variants of picromycin[14] and erythromycin[15] PKSs. Mechanistically, the iteration process poses a riddle because modular PKSs form homodimers. Insights into the protein structures and cross-linking experiments have revealed a double helical model of modular PKSs where the functional domains of the homodimer interact with each other.[16,17] In analogy to fatty acid biosynthesis, a polyketide chain is formed through the

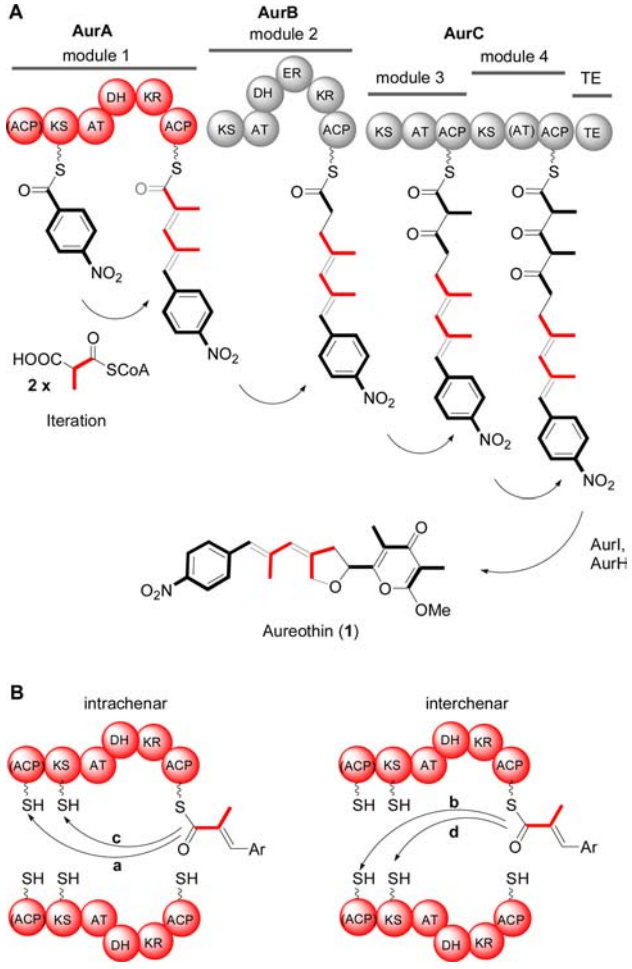

Figure 1. (A) Model aureothin biosynthesis involving an iterative type I PKS module (AurA); (B) possible mechanisms for intrachenar and interchenar retrotransfer of the ACP-bound intermediate (Ar = *p*-nitrophenyl).

interaction of both PKS strands. For the forward direction, the ketosynthase (KS) domain of chain A transfers the growing polyketide chain to the acyl carrier protein (ACP) domain of chain B, and vice versa. The malonyl extender unit is selected by the acyl transferase (AT) domain that can, in principle, load the ACPs of both monomers.[18] However, the mechanism of

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chain transfer in an iteration event is fully unknown. In principle, one may conceive the following scenarios (Figure 1B): the intermediate is transferred from the ACP domain of one PKS chain to the (a) ACP domain on the same PKS chain ('intrachenar'); (b) ACP domain on the opposite PKS chain ('interchenar'); (c) KS domain upstream on the same PKS chain ('intrachenar'); or (d) KS domain upstream on the opposite chain ('interchenar'). Here, we address this issue by mutational analysis of the PKS involved in the biosynthesis of aureothin, an antiproliferative and antifungal metabolite of *Streptomyces thioluteus*.[7]

Initially, we sought to monitor the intermediates generated by the iterative module on AurA. For this purpose, we cloned *aurA* and heterologously expressed it in *Streptomyces lividans*. Through high-resolution Orbitrap MS (Exactive) analysis, we monitored masses that correspond to polyketide intermediates that are prematurely released occurring after one and two rounds of elongations. To unequivocally determine their structures, we synthesized authentic standards of the postulated aureothin precursors **2** and **3** and compared their retention time and mass spectra with the observed metabolites. Thus, the proposed intermediates of AurA could be clearly identified (Figure 2).

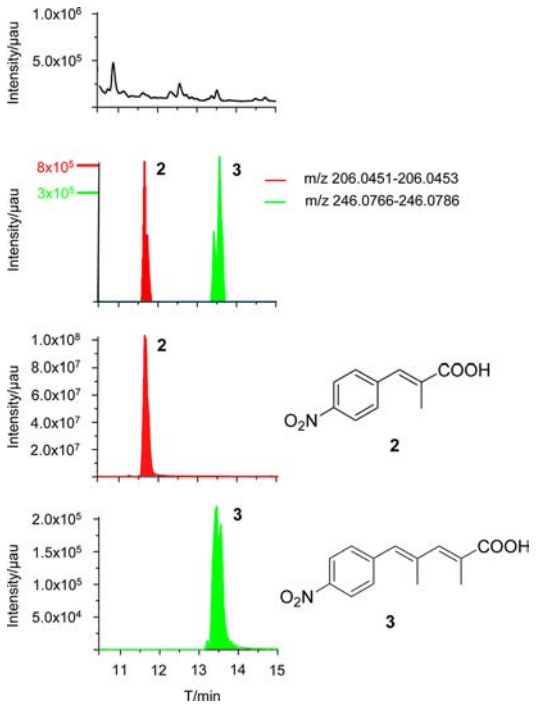

Figure 2. Detection of polyketide intermediates **2** and **3** in an *S. lividans* culture heterologously expressing *aurA*. UV trace (top, 254 nm) and ion extraction chromatograms (Orbitrap, negative mode) scanning for exact masses of **2** and **3**, and comparison with synthetic reference compounds.

For the systematic investigation of the mode of retrotransfer, we next aimed at combining variants of the iterative *aur* PKS module that are deficient in KS, AT, and ACP domains. As a prerequisite for this, it has been verified earlier in modular PKS that targeted mutations lead to a complete domain inactivation, but do not affect the conformation or folding of the protein.[19,20] Thus, it should be viable to reconstitute the iterative PKS module AurA from two mutated polypeptide chains, each lacking a critical PKS domain for chain propagation (KS, AT, or ACP). Through statistical recombination of two mutated monomers, three types of AurA dimers may form. These are the two functionally inactive "homodimers" lacking the minimal set of PKS domains, and one potentially active "heterodimer" bearing the complete set of catalytically active domains, yet distributed among two subunits (Figure 3).

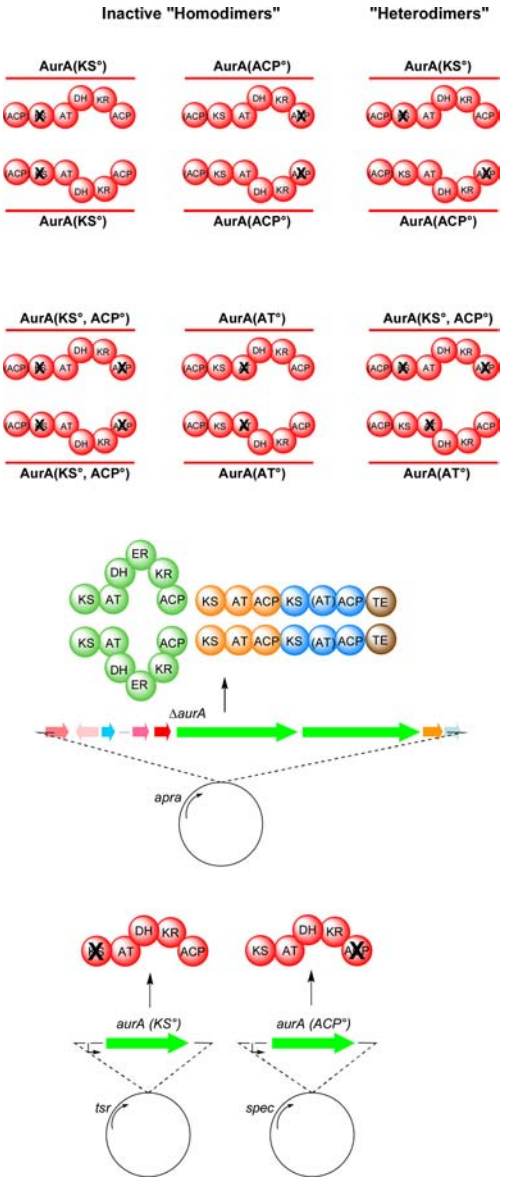

Figure 3. Outline of possible nonfunctional "homodimers" and potentially functional "heterodimers" of mutated AurA variants (top); sketch of three-plasmid-system for the reconstitution of the *aur* pathway and for mutagenesis of individual AurA subunits (bottom).

To warrant the native PKS function, we decided to employ the entire aureothin processing line in vivo, exchanging only the AurA part. An in vivo approach may be limited since one cannot rule out cross-complementation of functional units. However, various examples have shown that the function of PKS components may be altered when taken out of the natural context.[2] As a necessity for conducting mutational analyses of individual strands of the megasynthase we tested a flexible multivector host system composed of an integrative plasmid for

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the entire biosynthesis genes except *aurA* and two episomal plasmids for *aurA* variants. A Δ*aurA* mutant was successfully generated by PCR-targeting,[10] and in order to rule out polar effects resulting from gene deletion, the strain was complemented through coexpression of *aurA* on a free-standing plasmid (Figure 3).

For the targeted inactivation of the PKS domains, fragments of *aurA* were cloned and codons for critical amino acids were individually exchanged for alanine codons by site-directed mutagenesis. In the KS1 domain, the conserved cysteine in the DTACSSS motif was chosen. For the ACP1 domain, we targeted the conserved serine in the GFDSLR motif that serves as an anchor for the phosphopantetheinyl residue, and in the AT1 domain, the conserved serine in the GHSQG motif was replaced. In this way, we generated four constructs coding for AurA variants lacking one or two functional domains, AurA(KS°), AurA(AT°), AurA(ACP°), and AurA(KS°, ACP°). As to a potential ACP−ACP retrotransfer, we noted a potential phosphopantetheine binding site upstream of KS1. Because of the aberrant motif, however, we chose to truncate the N-terminus of AurA, yielding Aur(N°), to exclude the potential involvement of this region in the iteration process. The plasmids harboring the mutated *aurA* variants were then introduced into the expression host *S. lividans* and co-expressed with the remaining *aur* biosynthesis genes, and metabolite production was monitored by HPLC−MS (Figure 4). Since aureothin biosynthesis was restored in the Δ*aurA* mutant complemented with *aurA*(N°), we could readily exclude scenarios (a) and (b) involving ACP−ACP retrotransfers. This is intriguing since skipping in a modular PKS has been shown to involve an ACP−ACP transfer.[19] The decrease in aureothin production (20% of wild type) may be due to reduced solubility or change in protein folding of the mutated AurA variant. As negative controls for the domain complementation experiments, we first individually coexpressed the *aurA*(KS^0) and *aurA*(ACP^0) genes with the incomplete *aur* gene cluster. As expected, the individual domain mutations led to a complete abrogation of polyketide biosynthesis.[21] First, we explored the possibility of an intrachenar KS−ACP retrotransfer by coexpressing *aurA*(KS°, ACP°) and *aurA*(AT^0) in the Δ*aurA* mutant. Since aureothin formation could not be observed in the mutant broth, it appeared that homo- and heterodimers of these AurA variants were not capable of producing any polyketide intermediates. Next, to evaluate the viability of a KS−ACP retrotransfer from one PKS chain to the other (interchenar, scenario d), we co-expressed the *aurA*(KS^0) and *aurA*(ACP^0) variants using the Δ*aurA* mutant. In this case, aureothin production could be clearly detected. Although the production rate was substantially lower than in the wild type, this result is a clear indication for a successful complementation of the inactivated PKS domains in an AurA(KS°)−AurA-(ACP°) heterodimer. Aureothin production in this mutant unequivocally shows that one subset of PKS domains is used twice during the iteration event, and that the ACP-bound intermediate is transferred back to the KS located on the opposite PKS chain. The reduced production rate (15% of wild type) may be rationalized by less efficient AurA, since only one subset of domains can be used and the statistical probability of heterodimer formation.

In conclusion, through targeted domain mutagenesis, cross-complementation experiments, and metabolic profiling, we have addressed for the first time the course of iterative chain elongation in a type I polyketide synthase. We found that the *N*-terminus with tentative residual ACP function is not involved in the iteration process. Furthermore, we constructed functionally impaired modules that permit the exclusion of three of four possible mechanisms for intermediate retro-transfer. According to our data, the most likely scenario is (d), a retrotransfer of the biosynthetic intermediate from the ACP onto the KS domain located on the opposite polypeptide strand. This finding is not only of significance for understanding the mode of action of the large family of related bacterial multimodular megasynthases, but may also serve as model for the widely distributed fungal iterative type I PKSs. Future biochemical studies will help unraveling the exact mechanism of iteration.

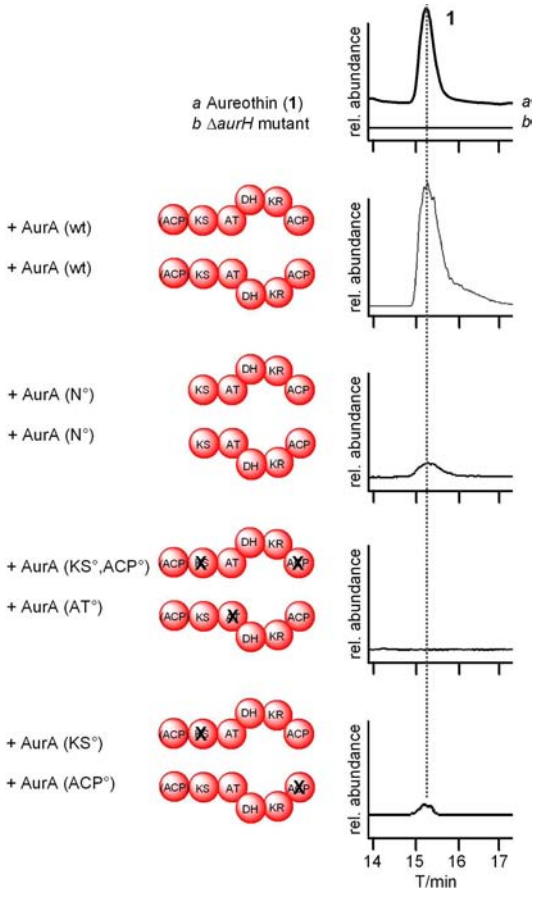

Figure 4. Key results of mutational analyses. HPLC/MS (SIM) profiles of Δ*aurA* mutant and complemented mutants.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental and analytical procedures, synthesis of reference compounds, additional HPLC profiles, description of mutant constructions and heterologous expression, domain analysis of *N*-terminal part of AurA. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

Christian.Hertweck@hki-jena.de

Notes

The authors declare no competing financial interest.

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