Polyketides

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Rational Design of Modular Polyketide Synthases: Morphing the Aureothin Pathway into a Luteoreticulin Assembly Line**

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Dedicated to Professor Manfred T. Reetz on the occasion of his 70th birthday

Abstract: *The unusual nitro-substituted polyketides aureothin, neoaureothin (spectinabilin), and luteoreticulin, which are produced by diverse Streptomyces species, point to a joint evolution. Through rational genetic recombination and domain exchanges we have successfully reprogrammed the modular (type I) aur polyketide synthase (PKS) into a synthase that generates luteoreticulin. This is the first rational transformation of a modular PKS to produce a complex polyketide that was initially isolated from a different bacterium. A unique aspect of this synthetic biology approach is that we exclusively used genes from a single biosynthesis gene cluster to design the artificial pathway, an avenue that likely emulates natural evolutionary processes. Furthermore, an unexpected, context-dependent switch in the regiospecificity of a pyrone methyl transferase was observed. We also describe an unprecedented scenario where an AT domain iteratively loads an extender unit onto the cognate ACP and the downstream ACP. This aberrant function is a novel case of non-colinear behavior of PKS domains.*

Many bacterial secondary metabolites that are currently in clinical use are produced by type I polyketide synthases (PKS). These giant enzymes are composed of diverse functional domains that govern the stepwise assembly of highly complex architectures.[1] To warrant control over the diverse biosynthetic steps, the growing chains are covalently tethered to the template, and the functional domains are organized into a series of modules.[2] Minimally, each module consists of a ketosynthase (KS), which catalyzes the fusion of activated acyl and malonyl units, and an acyl carrier protein (ACP), which serves as an anchor for the growing polyketide chain and binds the extender unit supplied by an acyl transferase (AT). The products of the condensation reaction may be processed by optional ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains, and eventually the final products are liberated by a thioesterase (TE) domain. Owing to their modular architecture and unidirectional chain propagation, in most bacterial type I PKS the size and functionalization of the polyketide backbone directly corresponds to the number and architecture of the PKS modules.[3] This principle of colinearity has provided the ground for predicting structures from PKS genes and for rationally reprogramming the biosynthetic code for complex polyketide pathways.[4] In nature, the various modular PKSs have evolved from horizontal gene transfer, gene duplications, recombinations, deletions, and mutations.[5] Yet, such evolutionary processes await emulation in the laboratory.

The structures of nitro-substituted pyrone metabolites neoaureothin (**1**; also known as spectinabilin), aureothin (**2**), luteoreticulin (**3**), and orinocin (**4**), which have been isolated from diverse *Streptomyces* spp., are highly suggestive of a common evolutionary origin (Figure 1).[6] Metabolites of this family have been shown to be potent antifungal, cytotoxic, antiviral, immunosuppressive, and antinematodal agents. Analyses of the biosynthesis gene clusters for aureothin (*aur*)[7] and its higher homologue neoaureothin (*nor*)[8] indicated that the *aur* PKS has evolved from the *nor* PKS by

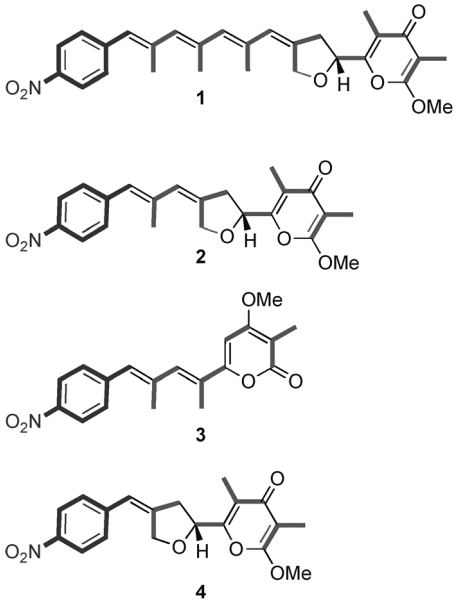

Figure 1. Structures of nitro-substituted pyrone metabolites from *Streptomyces* spp.

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gene deletion.[8] Interestingly, both assembly lines are exceptional owing to the iterative use of the first modules,[9] which break with the rule of colinearity.[10] The smallest congener (**4**), however, is not assembled from a smaller PKS, but results from photoinduced polyene splicing of neoaureothin.[11] Only the molecular basis for luteoreticulin biosynthesis has remained elusive, hampered by the lack of availability of a producing strain. Herein, we report the successful directed evolution of the aureothin PKS into an assembly line for its homologue luteoreticulin. Furthermore, we unveil a new case for non-canonical PKS programming and describe a surprising substrate-dependent switch of regiospecificity of a methyl transferase.

With the intention to design a modular PKS for luteoreticulin we compared the structures of aureothin and luteoreticulin. From the deduced biosynthetic code, we inferred that, in principle, the luteoreticulin core structure could be generated by an *aur* PKS variant lacking parts of the second and third modules (Figure 2). Specifically, the degree of β-keto processing and the choice of extender units needed to be changed. In addition, a methyl transferase with specificity for α-pyrones[12] would be required for the regiodivergent methylation. For the construction of the luteoreticulin backbone, we aimed at generating two types of truncated *aur* PKS, each lacking one (net) module. The resulting constructs would only differ in the origin and substrate specificity of the AT domain in module 2.

To construct the first PKS fusion (*lut*$^{\#}$), gene fragments encoding domains between AT2 and KS3 were deleted from the *aur* PKS genes *aurB* and *aurC*. Plasmid pHJ48, an *Escherichia coli-Streptomyces* shuttle cosmid bearing the entire *aur* locus, and specific subclones thereof, were employed for the gene manipulation.

The boundaries between the KS domains and KS-AT linkers in modules 2 and 3 were chosen for the fusion sites. By means of PCR targeting, the corresponding gene regions was substituted with a spectinomycin resistance cassette (*addA*) flanked by *Hpa*I restriction sites. After successful replacement of the targeted gene region in *E. coli*, the cassette was removed by digestion with *Hpa*I and re-ligated to generate a seamless fusion (Figure 2; see also the Supporting Information).

The recombinant expression plasmid (pYU9) was introduced into the heterologous host *S. lividans* ZX1, and the metabolic profile of the transformant (*S. lividans* ZX1:pYU9) was monitored by HPLC-MS, which indicated that the strain produced new pyrone compounds. The main metabolites, **5** (0.11 mg L^{-1}), and **6** (0.35 mg L^{-1}; Figure 3), were isolated from mycelium and culture broth of a 20 L-fermentation and purified by open column chromatography, followed by preparative HPLC. According to HRESI-MS data and NMR spectra, both **5** and **6** bear a hydroxyl group at the C7a position. NOESY experiments revealed that compound **6** is an *E*/*Z* isomer of **5** (see the Supporting Information).

UV spectra and high resolution LC-MS data suggested that *S. lividans*:pYU9 produces other luteoreticulin analogues, including non-hydroxylated variants. However, the extremely low production titers for these compounds prevented their full characterization. As an alternative, we aimed

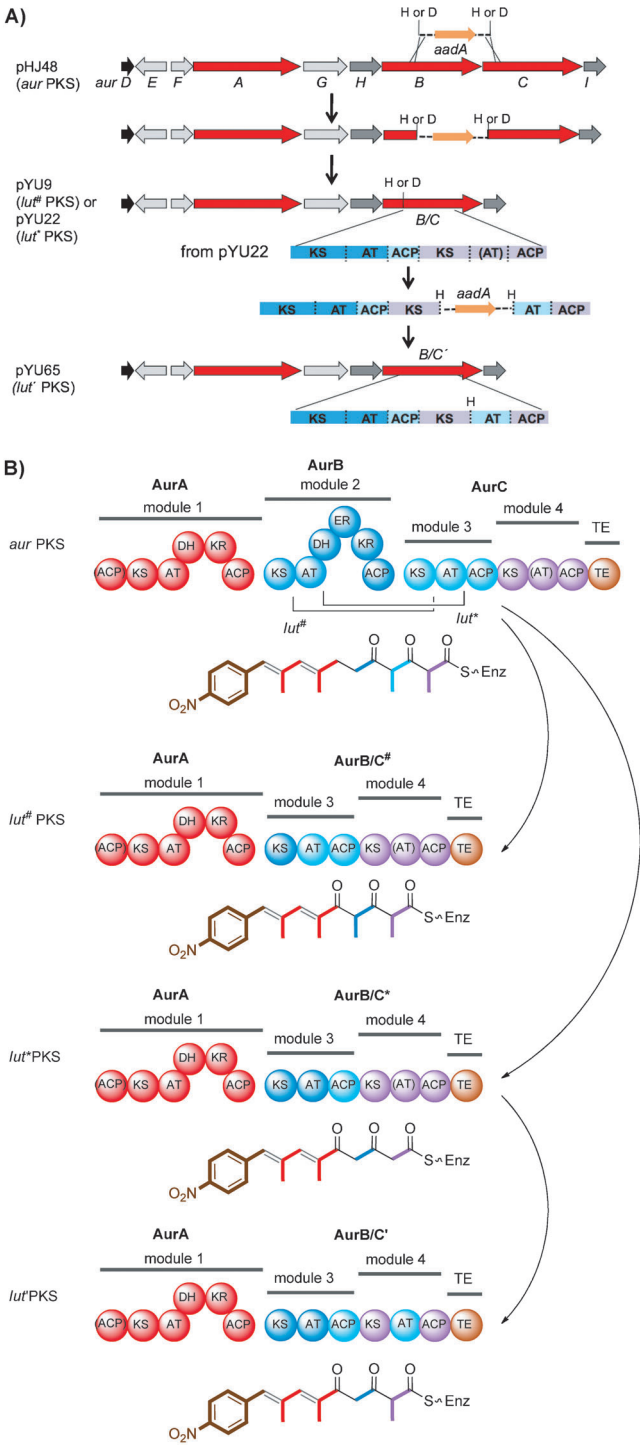


Figure 2. Overview of the strategy to generate the *lut*$^{\#}$ (pYU9), *lut** (pYU22) and *lut*′ (pYU65) PKS mutants. A) Engineered gene clusters. Deduced gene functions: AurA (PKS module 1), AurB (PKS module 2), AurC (PKS modules 3 and 4), AurD (transcriptional regulator), AurE (acyl-CoA ligase), AurF (*N*-oxygenase), AurG (PABA synthase), AurH (cytochrome P*450* monooxygenase), AurI (methyltransferase); *aadA* (spectinomycin and streptomycin acetyltransferase). H; *Hpa*I, D; *Dra*I. The gene fragment encoding for the non-functional AT4 (purple) was exchanged with the gene fragment encoding AT3 (light blue) from the *aur* PKS. B) Architectures of the modular aureothin (*aur*) PKS and derived assembly lines *lut*$^{\#}$, *lut** and *lut*′ and structures of PKS intermediates (deduced from isolated products). The color code refers to the KS involved in the Claisen condensation.

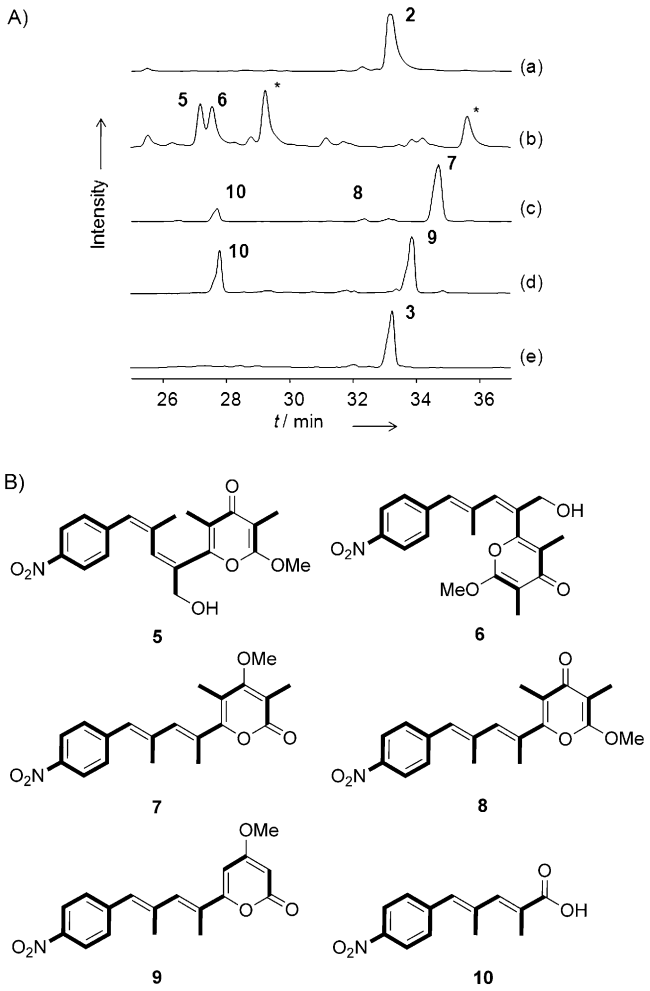


Figure 3. Monitoring of metabolic profiles for the wild-type producer and mutants. A) HPLC-MS analysis of extracts from: a) heterologous aureothin producer (*S. lividans* ZX1:pHJ48), b) mutant producing *lut*# PKS (*S. lividans* ZX1:pYU9), c) same as for (b), but lacking cytochrome P450 monooxygenase AurH (*S. albus*:pYU2), d) mutant producing *lut** PKS (*S. albus*:pYU22), and e) mutant producing *lut'* PKS (*S. albus*:pYU65.). Peaks marked with an asterisk derive from putative congeners that were not produced in the up-scaled fermentation and thus evaded structural elucidation. UV detection at 350 nm. B) Molecular structures of new metabolites isolated from engineered mutants (for structure elucidation, see the Supporting Information).

at blocking C7a hydroxylation. The most likely candidate for this reaction is the unusual cytochrome P450 monooxygenase AurH, which is responsible for the stepwise formation of the tetrahydrofuran ring from deoxyaureothin in the native pathway.[13] Yet, when alternative substrates are employed, AurH is capable of regiodivergent oxygenations.[14] To increase the yield of non-hydroxylated luteoreticulin analogues, the CYP monooxygenase gene (*aurH*) was deleted by PCR targeting, and the resulting construct (pYU2) was introduced into *S. albus*. To prevent *E*/*Z* isomerization, the cultivation and all downstream steps were performed in the dark. The main product (**7**; 0.11 mg L^{-1}) and a minor side product (**8**; 0.01 mg L^{-1}) were isolated from a 20 L culture of the *lut*# Δ*aurH* mutant (*S. albus*:pYU2), and their structures were determined by ^{1}H and ^{13}C NMR, HMBC, and HMQC. As desired, **7** and **8** lack the C7a hydroxy group, and show the correct double-bond configuration. Unexpectedly, the ^{13}C NMR shifts of the compounds revealed that main product **7** features an α-pyrone ring, whereas the minor congener **8** represents the γ-isomer. Thus, only the methylation pattern of the pyrone ring deviated from the target molecule.

To address this issue, we engineered an alternatively fused PKS (*lut**), in which the malonyl-specific *aur* AT2 was incorporated into the second module. In analogy to the recombination strategy utilized for fusion *lut*#, gene regions coding for domains between DH2 and AT3 were removed from the *aur* PKS genes with concomitant fusion of the *aurB* and *aurC* fragments (Figure 2). The C-terminal regions of AT2 and AT3, which are composed of homologous amino acids sequences, were chosen as fusion sites. The resulting plasmid (pYU22) was introduced into the heterologous host, and HPLC-MS profiling of the transformant culture indicated that a new luteoreticulin derivative (**9**; 0.12 mg L^{-1}) was produced by the *lut** PKS. The structure of **9** was fully elucidated by HR-MS and 1D- and 2D-NMR techniques. Interestingly, compound **9** proved to be a desmethyl variant of luteoreticulin, with an *O*-methylated α-pyrone ring (Figure 3). We also detected pathway intermediate (**10**) in the broths of both the *lut*# and the *lut** PKS mutants, which points to a premature release from the PKS downstream of AurA.

Compounds **7** and **9** differ from the target compound **3** in that they either lack a methyl group or have an extra one. The uniform methylation pattern on the pyrone rings suggests that the elongation unit for the final condensation step is governed by the AT domain in the penultimate module, not by the terminal AT domain. Indeed, the AT domain is substantially smaller than regular ATs, and lacks typical conserved motifs. Thus, we concluded that the terminal AT domain is nonfunctional. This is intriguing, as previous mutagenesis studies showed that the KS and ACP domains in the terminal module are necessary for aureothin biosynthesis. The only plausible explanation for this observation is that the extender unit used for the final elongation step is selected and loaded onto the terminal ACP by the penultimate AT (Figure 4, left). Yet, such iterative use of AT domains incorporated in PKS modules is without precedent.

To test this hypothesis, and to manufacture the methylation pattern found in luteoreticulin (**3**), we aimed at exchanging the terminal, non-functional AT domain in the

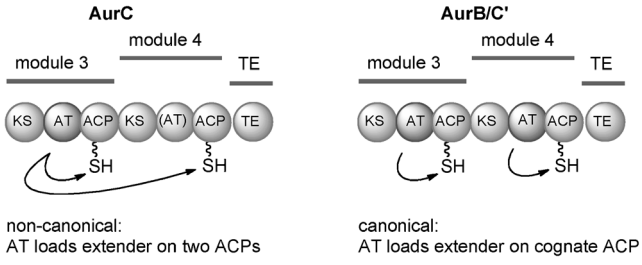
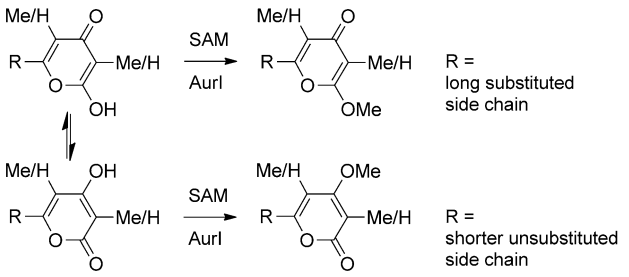


Figure 4. Non-canonical, iterative use of AT3, as in the modular *aur*, *lut*# and *lut** PKS vs. regular ACP loading by cognate ATs.

*lut** PKS for a functional AT domain, which loads methylmalonyl-CoA onto the adjacent ACP (Figure 2, bottom). To achieve this goal, we selected the AT3 domain of the *aur* PKS and introduced it into the terminal module of the *lut** PKS. For the recombination we used conserved motifs at the C-terminal regions of AT3 and AT4 of the *aur* PKS, as well as a boundary between KS4 and the N-terminus of the KS3-AT3 linker. A unique restriction site, *Hpa*I, was introduced into the corresponding gene fragment, and the DNA fragment coding the AT3 was exchanged with the targeted inactive AT gene fragment by genetic recombination. A spectinomycin resistance cassette was introduced into the *Hpa*I restriction sites and the cassette was removed by enzymatic digestion with *Hpa*I (Figure 2; see also the Supporting Information).

Finally, the resulting plasmid (pYU65) coding for the *lut*' PKS was introduced into *S. albus*. HPLC-MS analysis of the transformant culture (*S. albus:*YU65) indicated that a new metabolite with the expected mass was produced. The compound was isolated from a culture (20 L) and purified by chromatography. ^{13}C NMR, HMBC, and HMQC data unequivocally proved that the engineered pathway produces the desired compound, luteoreticulin **3** (0.12 mg L^{-1}; Figure 3). Notably, the engineered compound not only features the desired α-pyrone ring and polyketide backbone, but also shows the correct substitution pattern at the heterocycle.

The successful construction of a luteroreticulin assembly line from the *aur* PKS and accessory enzymes has various important implications. First, we describe for the first time that an AT domain is capable of iteratively loading extender units onto the cognate ACP and the downstream ACP. This aberrant domain function is a novel case of non-colinear PKS codes and a noteworthy addition to the body of knowledge on diverse PKS functions. Second, we observe a wholly unexpected, context-dependent switch in the regiospecificity of a methyl transferase. Apparently, the site of methylation of the pyrone tautomers depends on the size and substitution of the polyketide backbone (Scheme 1). Third, and most significantly, we have successfully reprogrammed the *aur* PKS into a synthase that generates luteoreticulin. The stepwise morphing proceeded through engineered *lut*$^{\#}$, *lut*$^{\#}$(Δ*aurH*), *lut** and *lut*' PKS mutants, which not only provided access to new luteoreticulin analogues, but also devised an avenue that likely emulates natural recombination processes.



Scheme 1. Switch in regiospecificity of methyl transfer catalyzed by AurI.

In conclusion, the genetic manipulation of biosynthetic pathways, in the sense of pathway engineering, combinatorial biosynthesis, or synthetic biology, has proven a valuable approach to generate structural analogues of known bioactive compounds. Whereas in several cases mutations of terpene cyclases have led to terpenes found in other pathways,[15] examples for altering a polyketide pathway to yield another known polyketide are scarce. Through gene recombinations and modification of tailoring steps, auramycinone[16] and tetracenomycin D1[17] have been obtained in artificial type II PKS systems in bacteria, and domain swapping in a fungal PKS yielded bassianin in lieu of tenellin.[18] Herein, we describe the first rational transformation of a modular PKS to produce a complex polyketide that was initially isolated from a different bacterium. A unique aspect of this study is that we exclusively used genes from a single biosynthesis gene cluster to design the artificial luteoreticulin pathway. Beyond unveiling non-canonical PKS programming in the *aur* PKS, this study illustrates that it is indeed possible to morph a given modular PKS to produce naturally occurring polyketides with different backbones.

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