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Biosynthetic Origin of the Rare Nitroaryl Moiety of the Polyketide Antibiotic Aureothin: Involvement of an Unprecedented *N*-Oxygenase

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Structural diversity of polyketide metabolites results from various programmed events dictated by polyketide synthases (PKSs), including the number of Claisen condensations, degree of reduction, and mode of cyclization.[1,2] While the vast majority of polyketides is primed with acetate or propionate, a number of PKSs utilize alternative starter units, such as short-chain branched fatty acids, alicyclic and aromatic acids, and amino acids.[3] The nature of the primer unit used often provides important structural and biological features to the molecule, and variation of the starter can significantly alter the activity profile of the natural product.[3,4] An impressive example for such an approach is the engineered biosynthesis of doramectin, a highly potent anthelmintic agent obtained by exchanging the avermectin isobutyrate starter for cyclohexanoate.[5]

In a structure-guided search for novel PKS primers, we were prompted by the peculiar structure of aureothin (aur, **1**), a rare nitroaryl-substituted metabolite from the soil bacterium *Streptomyces thioluteus*,[6] which exhibits antitumoral, antifungal, and insecticidal activities.[7] Interestingly, a closely related *N*-acetyl derivative **2**, recently isolated from *Streptomyces netropsis*,[8] has been found to be a highly selective agent against *Helicobacter pylori*, the most common cause of chronic gastritis (Figure 1). Presumably, the general cytotoxicity of **1** is attributable to the nitroaryl residue, and variation of the aromatic headgroup of **1** may be a promising strategy for drug discovery. The structure of aureothin and early feeding experiments suggest a mixed polyketide biosynthetic pathway. In fact, the incorporation pattern of ^{13}C-labeled precursors indicates that the aureothin backbone is formally assembled from one acetate and four propionate units, possibly by a type I PKS (Figure 1).[9] However, nature and origin of the aromatic nitro moiety have not yet been firmly established. The structure of the C_7N unit is very suggestive for *p*-aminobenzoate (PABA), or a derivative thereof, as a key intermediate in aureothin biosynthesis. Surprisingly though, so far all efforts to incorporate labeled PABA into **1** failed.[10,11] By ESI-MS analyses we have detected traces of *p*-nitrobenzoate (PNBA) in the crude extract of a *S. thioluteus* HKI-227 culture. To probe PNBA as potential PKS starter, d_4-PNBA (CDN Isotopes) was pulse-fed to a shaking culture of *S. thioluteus*, and the mycelium was harvested and extracted. In fact, MS analyses revealed that d_4-PNBA was incorporated at a fairly high rate (ca. 20%). The M+4 shift in molecule and daughter ions generated by MS^n experiments unambiguously indicates that PNBA acts as *aur* PKS starter unit.

We assumed that PNBA would be synthesized in *S. thioluteus* by oxidation of *p*-aminobenzoate (PABA) and detected two copies of PABA synthase genes by PCR in the *S. thioluteus* genome. One copy was homologous to PABA synthase genes involved in the biosynthesis of tetrahydrofolic acid. In primary metabolism PABA is synthesized from chorismate via 4-amino-4-deoxychorismate **4** by the successive action of two individual PABA synthase subunits, PabA and PabB.[12,13] The deduced protein of the other copy obtained by PCR resembled PABA synthases from eukaryotes and bacterial

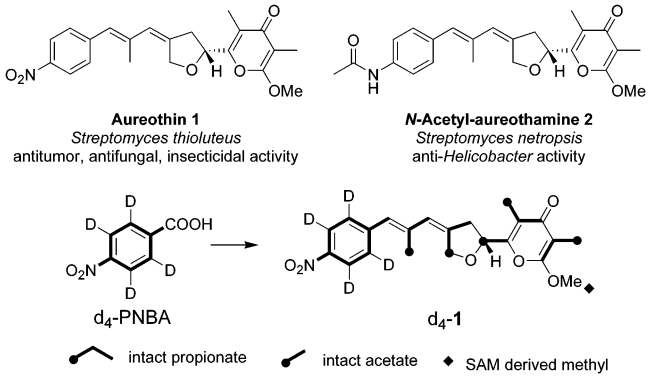

Figure 1. Structures of aureothin (**1**) and *N*-acetyl-aureothamine (**2**) and results of isotope labeling experiments.

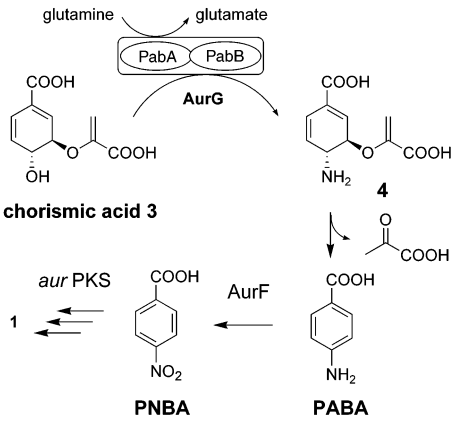

Figure 2. Biosynthetic model for PNBA biosynthesis.

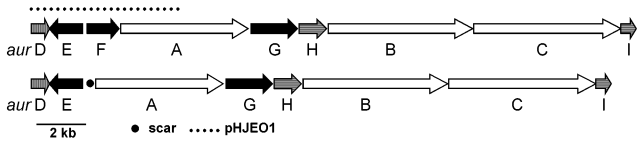

Figure 3. Organization of the aur gene cluster as in pHJ48 (top) vs *aurF* null mutant pHJ48Δ*aurF* (bottom).

secondary metabolism (e.g. biosynthesis of polyene macrolides candicidin[14,15] and FR-008[16]) where both PABA synthase subunits are covalently linked (Figure 2). This PABA synthase gene probe enabled the identification of the entire aureothin (*aur*) biosynthesis gene cluster (Figure 3, GenBank accession no. AJ575648). The identity of the *aur* gene cluster was established by heterologous expression of the entire gene set (pHJ48)[17] in *S. lividans* ZX1.[18] In accord with the predicted pathway, the PABA synthase gene, designated *aurG*, is flanked by genes encoding a type I PKS and enzymes catalyzing late biosynthetic steps. Strikingly, no obvious candidate for a gene encoding an "*N*-oxygenase" could be detected. To screen for the obscure *N*-oxidation activity, several mutants

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Table 1. Results of Expression and Inactivation Experiments

strain/mutant	*N*-oxidation activity	aureothin production
S. lividans ZX1	−	−
S. lividans ZX1::pHJ48	+	+
S. lividans ZX1::pHJE01	+	−
S. lividans ZX1/pHJ60	+	−
S. lividans::pHJ48Δ*aurF*	−	−
S. lividans::pHJ48Δ*aurF* + PNBA	n.d.	+

bearing truncated gene sets of the *aur* gene cluster were generated by successively truncating the 27 kb *aur* gene cluster on the shuttle plasmid pHJ48 and expression of the resulting gene sets in *S. lividans* ZX1,[18] a host that is not capable of oxidizing PABA. In a whole-cell approach, mycelium of 2-d-old cultures of the transformants was harvested, washed, and resuspended in phosphate buffer.[10] PABA was administered to both the transformants and the *S. lividans* ZX1 host, as well as the host bearing an empty vector, as negative controls. As monitored by TLC and MS, aureothin production was completely abolished in all transformants. However, *N*-oxidation activity was retained in *S. lividans*::pHJE01 harboring a ca. 6 kb genomic fragment, and the major portion of supplied PABA was readily transformed to PNBA within several hours. On the 6 kb fragment, a 1 kb open reading frame (*aurF*) was localized, which could not be assigned through database searches. The single gene was cloned downstream of the constitutive promotor *ermE** of pWHM4*,[19] yielding pHJ60. Expression of *aurF* in *S. lividans* ZX1 and probing the whole-cell preparation with the *N*-oxidation assay clearly indicated that the unknown gene encodes a novel *N*-oxygenase.

Despite their wide distribution among various organisms, aromatic nitro compounds are quite rare in nature, with chloramphenicol[20] and pyrrolnitrin[21] being among the most prominent examples. To date, not much is known about the biosynthesis of nitro aromatic natural products. In a few cases, aromatic nitro substitution has been discussed,[22] but oxidation of primary amines, which probably affords three steps via hydroxylamine and nitroso intermediates, appears to be more likely.[22] The, as yet, only example of an *N*-oxidizing enzyme involved in nitro group formation, an oxygenase with a Rieske motif, has been implicated in pyrrolnitrin biosynthesis.[23] Strikingly, the deduced 336-amino acid gene product of *aurF* did not show any homology to any known oxidoreductases, and neither common motifs nor cofactor-binding sites could be identified by database searches (BLAST, PROSITE).

Further evidence for the intriguing *N*-oxidation activity was obtained by in-frame deletion of *aurF* in pHJ48 (Figure 3, indicated by "scar"). A double-crossover experiment in *Escherichia coli* making use of the REDIRECT system[24] and transfer of the resulting plasmid (pHJ48Δ*aurF*) into *S. lividans* ZX1 yielded a mutant, in which both *N*-oxidation activity and aureothin production was abolished (Table 1). However, aureothin biosynthesis could be fully restored upon supplying the mutant culture with synthetic PNBA. This result clearly indicates that *N*-oxidation occurs before the C_7N unit is loaded onto the PKS and not during or after polyketide chain propagation. Moreover, deletion of *aurF* might serve as a means for mutasynthetic approaches toward novel aureothin derivatives. Conversely, deletion of the PABA synthase gene *aurG* does not appear suitable for this purpose, since PABA might be provided by the primary metabolism.

In conclusion, we have elucidated the biosynthetic origin of the rare nitro aryl moiety from the antibiotic aureothin using both molecular and chemical approaches. By incorporation of d_4-PNBA into aureothin we have shown for the first time that *p*-nitrobenzoate (PNBA) may serve as starter unit for a polyketide synthase. Cloning, expression and deletion of *aur*F revealed that PABA is transformed into the corresponding nitro compound by an unprecedented type of *N*-oxygenase. The novel PABA-oxidizing enzyme AurF provides a convenient model system to investigate the yet unresolved mechanism of nitro group formation, and might evolve as a valuable tool for more general chemo-enzymatic applications. Finally, the construction of the *N*-oxygenase null mutant not only sheds light on the timing of *N*-oxidation but also provides the framework for future mutasynthetic approaches toward aureothin derivatives with possibly altered bioactivity profiles.

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Supporting Information Available: Isotope labeling experiments with LC−MS data, *N*-oxidation assay, expression of *aurF*, construction of *aurF* null mutant (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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