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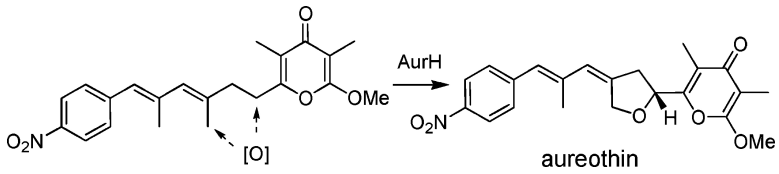


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# Formation of the Aureothin Tetrahydrofuran Ring by a Bifunctional Cytochrome P450 Monooxygenase

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Cytochrome P450 monooxygenases (CYT P450) represent a valuable group of biocatalysts that are capable of introducing oxygen functionalities into nonactivated carbons.[1,2] Many of these enzyme-catalyzed reactions are difficult or yet impossible to emulate using classical synthetic methods.[3] In polyketide biosynthesis, P450-mediated tailoring reactions can have a marked influence on the activity profiles of the resulting metabolites and thus represent important targets for pathway engineering.[4] While most P450 oxygenases are monofunctional, catalyzing a single hydroxylation or epoxidation of a substrate, multifunctional CYT P450s are rare and little explored. From a mechanistic and practical viewpoint, there is a particular interest in P450 oxygenases that would catalyze, for example, the stereospecific formation of substituted medium-ring oxygen heterocycles.[5] Such an intriguing structural feature is represented by the exomethylene tetrahydrofuran moiety of aureothin (**1**), a polyketide metabolite of *Streptomyces thioluteus* with antifungal, cytotoxic, and insecticidal activities (Figure 1).[6−8] A similar substructure can also be found in the avermectin family of anthelmintic and insecticidal compounds, where the cytochrome P450 AveE has been implicated to take part in furan ring formation. However, the function of AveE has not been firmly established to date.[9,10]

The biosynthesis of aureothin (*aur*), as revealed by labeling experiments[11,12] and by analysis of the entire *aur* biosynthesis gene cluster,[13] involves the assembly of the polyketide backbone from an unprecedented *p*-nitrobenzoate (PNBA) starter unit and five (methyl)malonyl-CoA extenders by an aberrant iterative modular polyketide synthase (PKS).[13] After the polyketide backbone is formed, two tailoring reactions are required for completion of the biosynthesis, including pyrone methylation by AurI and formation of the homochiral five-membered heterocycle. By sequence analysis and functional studies, two oxygenase genes, *aurF* and *aurH*, were detected in the *aur* gene cluster.[13] Heterologous expression and inactivation revealed that AurF is solely involved in the synthesis of the PKS primer, i.e., oxidation of *p*-aminobenzoate to *p*-nitrobenzoate.[12] Thus, the best candidate gene for the post-PKS steps is AurH. The deduced 406 aa gene product of *aurH* shows a clear P450 signature (Pfam: COG2124) with the highly conserved DxPxHxRxR and FGxGxHxCLG motifs, the latter of which is involved in heme binding. AurH is most similar to a novel class of hydroxylases from *Mycobacterium smegmatis* (36% identity), which are employed for N-heterocycle degradation.[14] Surprisingly, AurH shares much lower homology with functionally related P450 monooxygenases involved in polyketide tailoring, such as 6-deoxyerythronolide B hydroxylase EryF from *Saccharopolyspora erythraea* (28% identity)[15] and even AveE from *S. avermitilis* (13.5% identity).[9,10] To prove the involvement of AurH in the unusual oxidative heterocyclization we aimed at inactivation. Since to date all attempts to introduce DNA into the wild-type strain failed, an *Escherichia coli*−*Streptomyces* shuttle cosmid bearing the entire *aur* biosynthesis gene cluster, pHJ48,[13] appeared ideally suited for

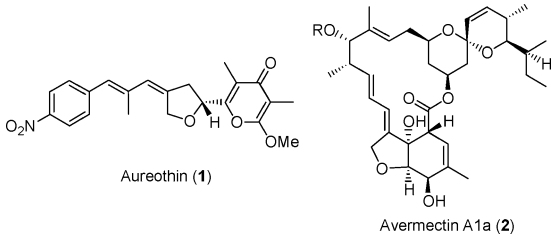


***Figure 1.*** Structures of aureothin (**1**) and avermectin A1a (**2**).

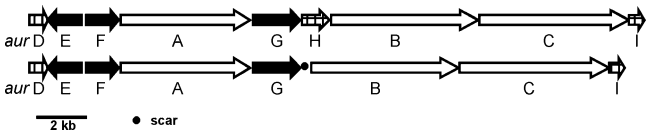


***Figure 2.*** Organization of the *aur* gene cluster as in pHJ48 (top) vs *aurH* null mutant pHJ98 (bottom).

generating knock out mutants in *E. coli* by recombination with the λ-red system.[16] After excision of *aurH* from the *aur* gene cluster and confirmation of the deletion by restriction mapping, the resulting cosmid (pHJ98, Figure 2) was introduced into protoplasts of *Streptomyces lividans* ZX1,[17] an expression host that was used previously for polyketide production.[13]

The resulting transformant, *S. lividans* ZX1::pHJ98, was selected for apramycin resistance and cultivated. TLC, HPLC, and MS analyses of the crude extract from a plate culture revealed that in lieu of aureothin another fluorescent metabolite with slightly lower polarity was formed. The structure of the new metabolite was fully resolved after purification of decent quantities (89 mg) from mycelium of a 20 l *S. lividans* ZX1::pHJ98 fermentation. HR-MS and $^{13}C$ NMR data account for a molecular composition of $C_{22}H_{25}NO_5$. All NMR signals were fully assigned by DEPT135, HMQC, and HMBC experiments, unequivocally revealing the structure of deoxyaureothin (**4**), as shown in Figure 3. HMBC coupling of C2 with the methoxy methyl reveals a γ-pyrone arrangement. In addition, the all-trans configuration of the diene was firmly established by NOE measurements. The same structure as **4** has already been proposed for luteothin, a metabolite of *Streptomyces luteoreticuli*, unfortunately without providing spectroscopical evidence.[18,19] The antimicrobial profile of deoxyaureothin is very similar to aureothin, albeit with slightly reduced antifungal activity. Strikingly though, deoxyaureothin exhibits a 5-fold increased cytotoxic activity against human K-562 leukemia cells ($IC_{50}$: 5 mM vs 25 mM of **1**).

In the Δ*aurH* mutant, aureothin biosynthesis was fully restored upon coexpression of *aurH*, which has been amplified by PCR and cloned downstream of the constitutive *ermE* promotor in pWHM4*,[20] yielding the self-replicating expression plasmid pHJ110. While these inactivation and complementation experiments clearly demonstrate that AurH is required for furan ring formation, it needed to be established if this novel CYT P450 is indeed sufficient for catalyzing


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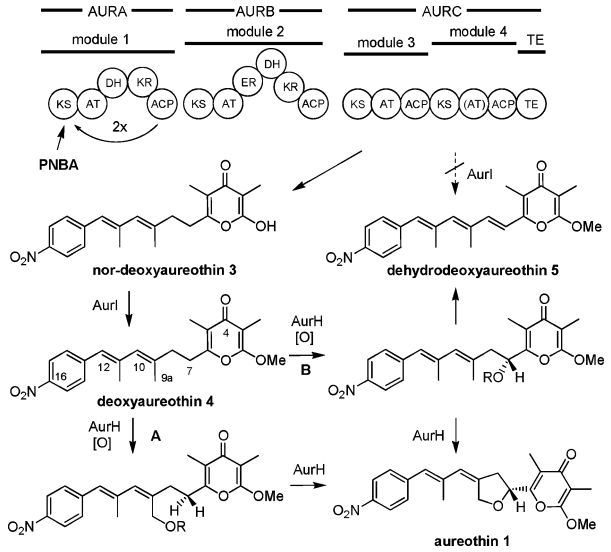


***Figure 3.*** Model of aureothin (**1**) biosynthesis via deoxyaureothin (**4**). The final ring closure might occur through a radical mechanism or through nucleophilic substitution via a bis-hydroxylated intermediate.

the formation of both C−O bonds of the furan moiety. For this purpose, AurH was heterologously expressed in *S. lividans* ZX1 using expression plasmid pHJ110. Deoxyaureothin was administered to a culture of *S. lividans* ZX1/pHJ110 and a control strain bearing the empty vector only. While the strain expressing AurH readily transformed deoxyaureothin into aureothin, as monitored by LC−MS, the control strain did not show any sign of biotransformation.

Synthesis of the furan ring represents the last step in the aureothin pathway, which is further supported by inactivation of the methyl transferase gene *aurI*, yielding *nor*-desoxyaureothin (**3**) but not *nor*-aureothin (He, J.; Hertweck, C., unpublished data). Obviously, this final biosynthetic step occurs with the subsequent oxidation of two nonactivated carbons, and thus, two scenarios are generally conceivable for this rare oxidation sequence: either the allylic position (9a-C) is attacked first (route A, Figure 3) or the methylene (7-C) adjacent to the pyrone ring (route B), setting the stereochemistry of the resulting heterocycle at an earlier stage. Significant support for route B is provided by the occurrence of traces (<20 $\mu$g/L) of another nitrobenzoate-primed polyketide metabolite that is produced by *S. thioluteus* and the host expressing the entire set of genes (*S. lividans* ZX1::pHJ48), but not by the Δ*aurH* mutant. From HR-MS, $MS^n$, IR, and $^1H$ NMR data, the structure of deoxydehydroaureothin (**5**) was deduced.

In general, **5** could be the result of an error-prone $\beta$-keto processing of the second module (AurB), where the ER domain of module 2 was accidentally skipped (Figure 3). Strikingly though, the triene could not be detected in the Δ*aurH* mutant (*S. lividans* ZX1::pHJ98), where the identical PKS is employed. It is thus more plausible that **5** is a shunt product from route B, resulting from hydroxylation and subsequent dehydration (Figure 1). Furthermore, biotransformation experiments with heterologously expressed AurH revealed that **5**, where the 9a-C methyl group is available, but the 7-C methylene is masked, is in fact not a substrate of AurH. The absence of any oxygenated derivatives of **5** could be another indication that oxygenation is not initiated at the methyl group. Since AurH contains only a single heme-binding motif, both C−O bond formations must be catalyzed at the same active site. However, the carbons that are oxidized are sufficiently close, which would only require small changes of substrate-binding orientation. Oxidations at multiple sites catalyzed by single CYT P450s have been reported for eukaryotic terpene biosynthesis.[21] To our knowledge, the results presented here provide the first evidence for a single CYT P450 catalyzing a two-step heterocyclization.

In conclusion, we have demonstrated that AurH is a multifunctional cytochrome P450 monooxygenase catalyzing the formation of the homochiral furan ring of aureothin, which constitutes the last step in the biosynthetic pathway. Inactivation of *aurH* yielded a mutant producing deoxyaureothin, a metabolite that exhibits a significantly improved cytotoxicity against leukemia cells compared to aureothin. Complementation of the mutant and biotransformation of deoxyaureothin by heterologously expressed AurH restored aureothin biosynthesis and provided the strongest evidence that a single P450 monooxygenase is capable of subsequently introducing oxygen at two nonactivated carbons. Finally, detection of an oxygenation shunt product allows the first glance into this intriguing reaction sequence. Further studies will disclose the potential of AurH as novel biocatalyst for the asymmetric (bio)synthesis of substituted furans.

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**Supporting Information Available:** Construction of Δ*aurH* mutant, fermentation procedures, spectroscopic data, complementation of the mutant and expression of *aurH*, and cytotoxicity assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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