Biosynthesis

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Sequential Asymmetric Polyketide Heterocyclization Catalyzed by a Single Cytochrome P450 Monooxygenase (AurH)**

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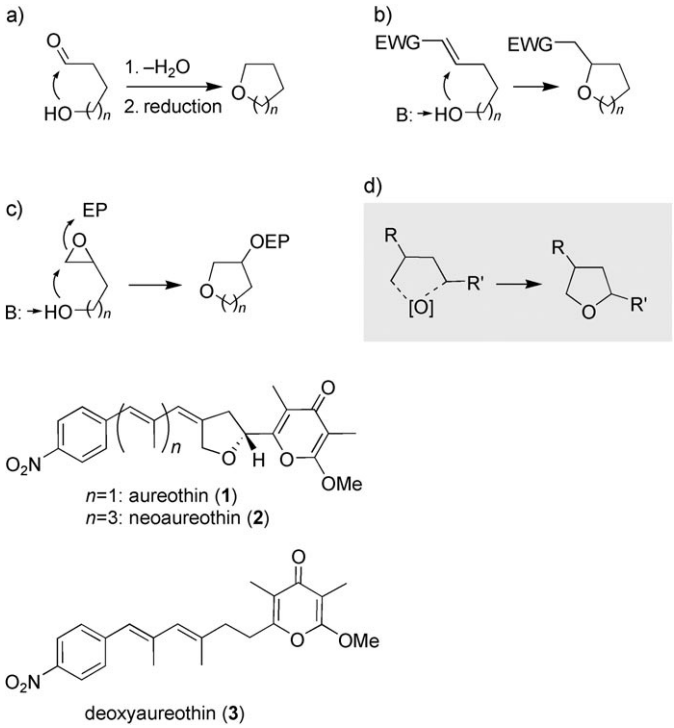
In memory of Joe Spencer

Complex polyketide metabolites, such as macrolides, polyenes, and polyethers, are an important source of novel therapeutics or serve as inspiration for their development.[1] Many of these compounds feature medium-sized heterocycles that lend rigidity to the carbon backbone and thus support a defined spatial arrangement of the substituents. Various biosynthetic routes to O heterocycles have been reported. In macrolides, pyran or tetrahydrofuran (THF) rings often result from spontaneous acetal formation. This reaction may be followed by dehydration and reduction, as proposed for the biosynthesis of kendomycin (Scheme 1 a).[2] The THF rings of nonactin, on the other hand, result from the enzyme-mediated conjugate addition of hydroxy groups to an adjacent acryloyl moiety (Scheme 1 b).[3] An alternative strategy for the synthesis of medium-sized O heterocycles is the nucleophilic ring opening of epoxides, a process that may account for THF formation in the linear kirromycin molecule.[4] This reaction has been implicated previously in the biosynthesis of polyethers, such as monensin,[5] nigericin,[6] nanchangmycin,[7] and tetronomycin,[8] whereby multiple cyclization reactions are directed by a zipper reaction involving a polyepoxide intermediate (Scheme 1 c).[9,10] The biosynthesis of the structurally intriguing polyether ladders found in marine toxins such as maitotoxin is probably based on the same principal route.[11] Various other routes are conceivable that involve as yet unveiled oxygenation reactions, such as in the aflatoxin biosynthetic pathway.[12] Even so, it is evident that the synthetic concepts outlined in Scheme 1 a–c cannot explain the formation of the tetrahydrofuryl moiety of the antitumor and antifungal natural product aureothin (**1**) and related pyrones.



Scheme 1. a–c) General cyclization mechanisms towards medium-sized O heterocycles in the biosynthesis of complex polyketides versus d) oxidative furan formation in the biosynthetic pathways of aureothin (**1**) and neoaureothin (**2**), and structure of the aureothin precursor **3**. EWG electron-withdrawing group, EP electrophile, B: base with a free pair of electrons.

Molecular analyses of the aureothin (**1**) and neoaureothin (**2**) biosynthetic pathways revealed that the nitroaryl-substituted polyketide chains are assembled from *p*-nitrobenzoate[13] and malonyl building blocks by noncanonical modular type-I polyketide synthase (PKS)[14–16] and tailored by methylation and oxygenation reactions.[17,18] We showed by gene deletion and complementation experiments that THF formation is correlated with the presence of a gene (*aurH*) that codes for a putative cytochrome P450 monooxygenase.[18] The Δ*aurH* mutant produces deoxyaureothin (**3**), which can be transformed into **1** by a *Streptomyces* host strain that expresses *aurH*. Although the in vivo experiments provided only limited and indirect information on the course of the enzymatic transformation, the THF group clearly results from the formation of two C–O bonds (Scheme 1 d). Herein we provide direct experimental evidence that the formation of the heterocycle is catalyzed by a single enzyme both in vivo and in vitro and reveal the reaction sequence and stereochemical course of this unprecedented oxygenation.

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To prove that AurH alone is sufficient for THF formation, and to learn more about this unusual oxygenation–heterocyclization reaction, we first aimed to transform deoxyaureothin (**3**) into **1** in vitro. For this purpose, we amplified the *aurH* gene from a plasmid containing the whole *aur* biosynthesis gene cluster and cloned it into a tailor-made expression vector pMR21 (see the Supporting Information). By using this vector, we succeeded in producing a soluble maltose-binding fusion protein with a cleavage site for factor Xa protease between N-terminal MBP (maltose-binding protein) and C-terminal AurH. Traceless proteolytic cleavage at this site produced native AurH. We produced the protein on a large scale by high-cell-density cultivation of *E. coli* BL21-(DE3)[19] containing plasmid pMR21, with induction by isopropyl β-D-1-thiogalactopyranoside (IPTG) and supplementation with 5-aminolevulinate, which supports heme biosynthesis in *E. coli*. Clarified cell extracts were passed through amylose and anion-exchange columns and fractionated. Factor Xa proteolysis and subsequent size-exclusion chromatography (SEC) yielded 15 mg of native AurH.

The in vitro activity of AurH was restored by a multienzyme electron-transport system consisting of spinach ferredoxin (10 μM), spinach ferredoxin–NADP oxidoreductase (0.1 units mL^{-1}), NADPH (1 mM), baker's yeast glucose-6-phosphate dehydrogenase (1 unit mL^{-1}), and glucose-6-phosphate (10 mM) at 30 °C. The oxygenation reaction was quenched by extraction with ethyl acetate and monitored by HPLC–MS. HPLC–MS profiling of the reaction mixture and comparison with an authentic reference indicated the slow formation of aureothin and the notably quicker formation of a new, more hydrophilic compound, **4** (Figure 1a). From UV and HRMS data it appeared that **4** was a monohydroxylated derivative of **3**. We assumed that this unknown compound was an intermediate or shunt product that resulted from an incomplete electron transfer or suboptimal reaction conditions. As the minute amounts of the new compound yielded by the in vitro biotransformation were not sufficient for complete structural elucidation, we had to consider another source. We had observed the formation of putative monohydroxylated compounds previously when non-natural substrates with an altered substitution pattern were used as substrates for AurH in vivo. Apparently, the monohydroxylation occurs only when the substrate is not capable of precise docking within the active site of the oxygenase. In stark contrast, the natural substrate **3** was transformed readily by AurH into the corresponding tetrahydrofuran **1**, and it seemed impossible to trap the proposed intermediate.

We thus sought to reverse the scenario by using the functionally equivalent oxygenase NorH from the homologous neoaureothin biosynthetic pathway.[16] Although the heterocyclization reaction in the neoaureothin pathway is mechanistically identical, **3** is shorter than the natural substrate, and thus docking is impaired. We anticipated that the oxygenation would be halted before the THF ring was formed. To test this hypothesis, we amplified the *norH* gene from a cosmid that harbors a major part of the *nor* biosynthesis gene cluster and cloned it downstream of the constitutively active permE promotor in pWHM4* to yield pNT85. We then expressed *norH* in a heterologous host for an

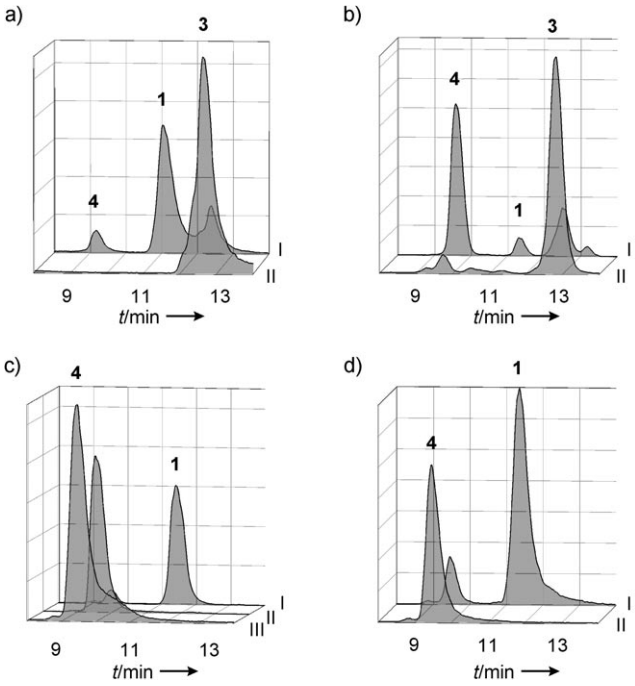
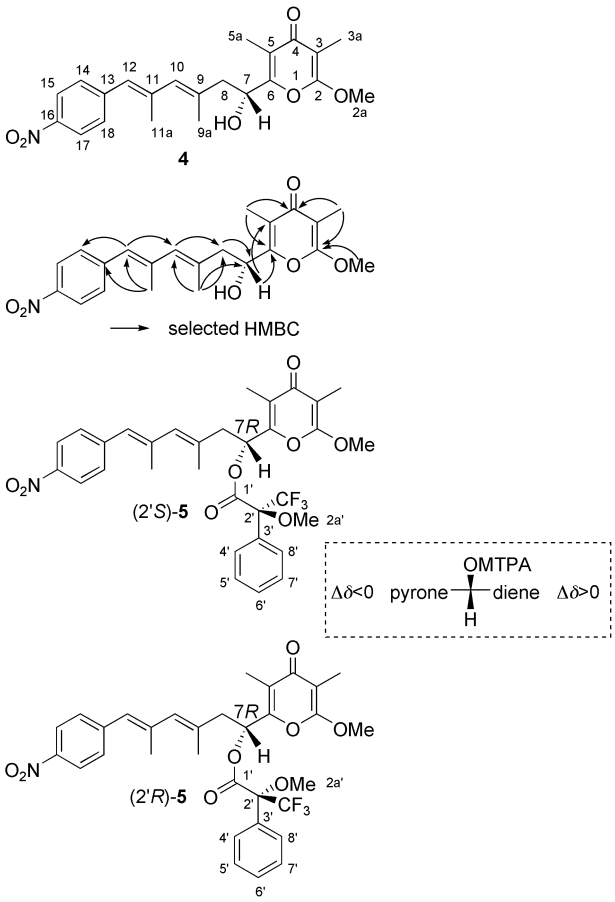


Figure 1. LC–MS profiles (selected-ion monitoring) of biotransformation reactions. a) I) In vitro oxygenation of **3** by AurH; II) negative control with denatured AurH (99 °C); b) I) in vivo transformation of **3** by NorH with *S. albus*/pNT85; II) negative control with *S. albus*/pWHM4*; c) I) in vivo biotransformation of **4** by AurH with *S. albus*/pHJ110; II) negative control with *S. albus*/pWHM4*; III) reference for **4**; d) I) in vitro oxidation/heterocyclization of **4** by AurH; II) negative control with denatured AurH (99 °C).

in vivo biotransformation of **3** and found that the same monohydroxylated compound **4** was produced (Figure 1b). For the production of **4**, we cross-complemented a Δ*aurH* mutant (*S. albus*:pHJ68) with *norH* by using a modified expression plasmid that included an oriT[20] for conjugation (pMR17). We analyzed the culture broth of *S. albus*:pHJ68/pMR17 by HPLC–MS and found that the metabolite produced was indeed identical to the compound formed in the in vitro assay (Figure 1b). To fully elucidate the structure of the new aureothin derivative, we subjected the crude extract of a fermentation on a 1 L scale to open column chromatography (silica, sephadex) and preparative HPLC to yield pure **4** (58 mg). The HRMS data and ^{13}C NMR spectrum of **4** indicated a molecular formula of $C_{22}H_{26}NO_6$. All spectroscopic and spectrometric data were in full agreement with a monohydroxylated derivative of **3** (Scheme 2). NMR spectroscopic data from 1D and 2D experiments provided clear evidence that the hydroxy group is connected to C7 (Scheme 2), and the splitting of the signals for the methylene hydrogen atoms at C8 indicated an adjacent stereogenic carbon center. The absolute configuration of the optically active secondary alcohol **4** ($[\alpha]_D^{22} = -23\ deg\,cm^3\,g^{-1}\,dm^{-1}$ in $CHCl_3$) was determined by the Mosher method according to a modified protocol.[21] A correlation of the NMR chemical-shift data[22] of (2′*S*)-**5** and (2′*R*)-**5** (see the Supporting Information) strongly suggested the 7*R* configuration, which



Scheme 2. Structure of **4** as confirmed by selected COSY and HMBC correlations. Structures of the diastereomeric Mosher esters (2′*S*)-**5** and (2′*R*)-**5**. MTPA = methoxy(trifluoromethyl)phenylacetyl.

is in full agreement with the configuration of the THF ring of **1**.[23]

We next investigated whether **4** is in fact an intermediate in the biosynthetic pathway to **1** or whether it is just a shunt product of an incomplete oxygenation reaction. For this purpose, **4** was administered to *S. albus*/pHJ110 (*aurH*). In fact, the alcohol **4** was completely converted into **1**, as evidenced by HPLC monitoring of the fermentation (Figure 1c). To prove unequivocally that AurH alone catalyzes the heterocyclization reaction, we repeated this biotransformation in vitro. Again, LC–MS monitoring clearly demonstrated that AurH catalyzes the formation of the aureothin THF moiety (Figure 1d). Finally, we attempted to elucidate the course of the entire tandem oxygenation reaction in vitro. Thus, we first optimized the reaction conditions and monitored the reaction by LC–MS at selected time intervals (Figure 2). After only 1 min, a peak corresponding to the monohydroxylated compound **4** was detectable. This peak reached a plateau within the first 10–20 min. The peak for the product **1** was visible after 20 min and grew in size, while the peak for starting material decreased, and the size of the peak for **4** remained constant.

All of these results clearly support a model according to which both C–O bonds are installed by a single enzyme in a

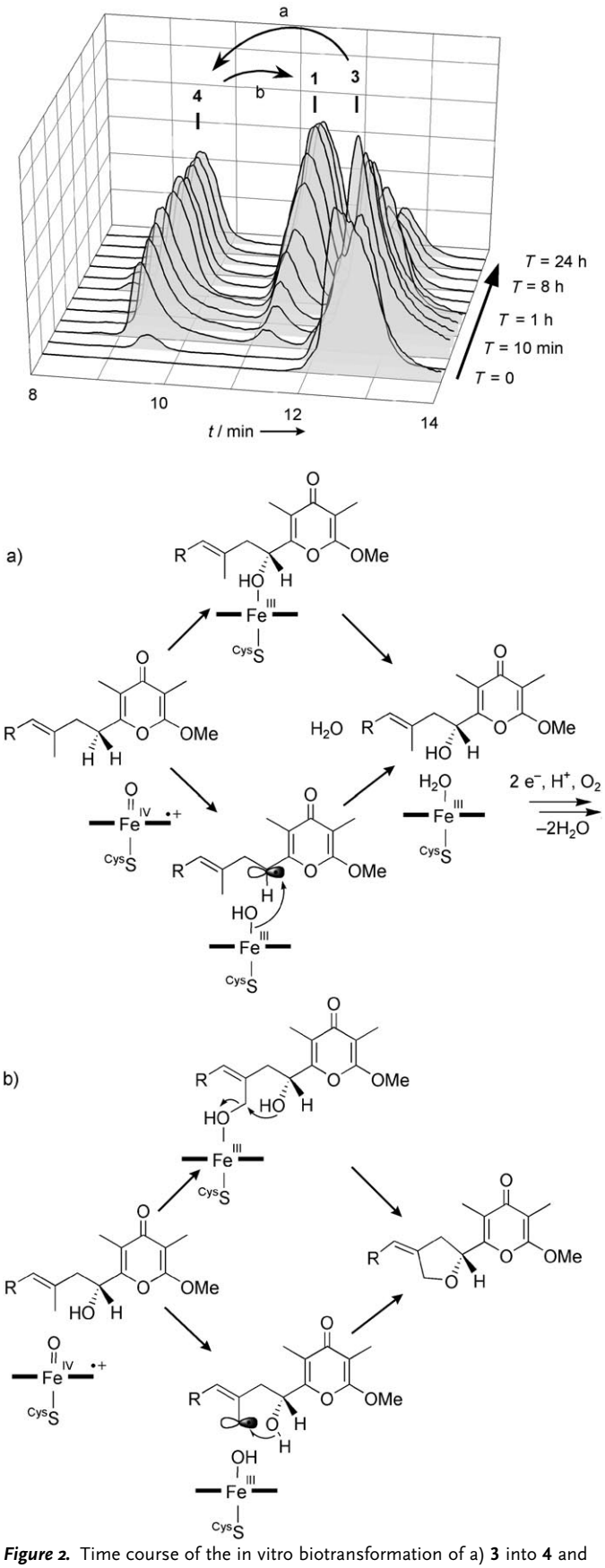


Figure 2. Time course of the in vitro biotransformation of a) **3** into **4** and b) **4** into **1** by the cytochrome P450 monooxygenase AurH, and model for sequential furan formation. LC–MS (selected-ion monitoring): $T = 0$, 1, 10, 20, 30, 45 min, 1, 2, 4, 8, 14, 24 h.

consecutive manner. The methylene position adjacent to the pyrone ring (C7) is attacked first (Figure 2, route a), which defines the absolute configuration of the resulting heterocycle. The second allylic position (C9a) is then oxygenated by the adjacent hydroxymethyl group (Figure 2, route b). Such a sequential heterocyclization reaction catalyzed by a single cytochrome P450 monooxygenase is unprecedented. Despite the known versatility of this class of enzymes,[24] until now only single hydroxylation or simple epoxidation reactions have been reported.[25,26] The multifunctional P450-1 enzyme from the gibberrelin pathway is a rare exception.[27]

The unprecedented sequential hydroxylation–heterocyclization sequence catalyzed by AurH can be rationalized on the basis of our current understanding of hydroxylation reactions mediated by cytochrome P450.[28,29] Accordingly, the C–O bond-formation reactions might involve a substitution reaction at a positively polarized allylic carbon atom or require an intermediary radical route (hydrogen abstraction–oxygen rebound). The detailed enzyme mechanism will be the subject of future studies. It should be noted that ether formation has been reported for non-heme iron oxygenases, such as hyoscyamine 6β-hydroxylase.[30]

In conclusion, we have overproduced and purified the unusual cytochrome P450 monooxygenase AurH in *E. coli* and reconstituted its activity in vitro. We have demonstrated through successful in vitro biotransformations that a single cytochrome P450 monooxygenase is capable of installing two C–O bonds sequentially. By trapping a chiral hydroxy intermediate and elucidating its absolute configuration we have established the order of the oxygenation reactions as well as the stereochemical course of the reaction. The unusual heterocyclization is likely in accord with current cytochrome P450 enzyme models. Even so, AurH is a novel biocatalyst that promotes a biotransformation that is as yet impossible to emulate by synthetic methods and that is unknown for other enzymes.

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