Biosynthesis

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A Semipinacol Rearrangement Directed by an Enzymatic System Featuring Dual-Function FAD-Dependent Monooxygenase**

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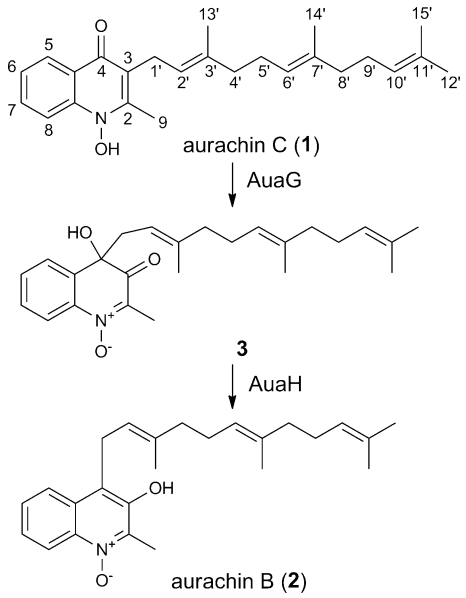
Nature has invented ingenious ways to biosynthesize biologically active small molecules that have been applied ever since to benefit human life in various ways. During the underlying biosynthetic processes, highly elaborate chemical reactions are often catalyzed by enzymatic systems, thereby enabling transformations under physiological conditions that would require harsh conditions or are hardly possible without enzymatic catalysis. Consequently, understanding novel biochemical transformations is of importance to eventually apply the knowledge gained to generate molecules of interest.

Aurachins are quinoline alkaloids isolated from the myxobacterium *Stigmatella aurantiaca* Sg a15; they have various biological activities, including antibacterial, antifungal, antiplasmodial, and mitochondrial respiration inhibition properties.[1–6] The biosynthesis of aurachin derivatives includes several interesting features in which the most intriguing reaction is the conversion of aurachin C (**1**) to B (**2**). This step involves the migration of the prenyl group from position C3 to C4, probably via a pinacol type rearrangement (Scheme 1).[7,8] Pinacol rearrangements are proposed to occur during the biosyntheses of various secondary metabolites, including aflatoxin B1, (+)-liphagal, (+)-asteltoxin, brevianamides, paraherquamide B, verscicolamide B, and notoamides.[9–11] However, no such biosynthetic hypotheses has been biochemically proven, although the proposed pathways



Scheme 1. Biosynthetic pathway of aurachin B (**2**) from aurachin C (**1**).

in turn inspired biomimetic approaches for natural product synthesis.[9] Therefore, an enzymatic system for pinacol-type rearrangement remained to be discovered in the biosynthesis of secondary metabolites. Even in primary metabolism there are only two reported examples in course of the biosynthesis of branched chain amino acids and 1-deoxy-D-xylulose-5-phosphate.[12–17]

The biosynthetic conversion of aurachin C (**1**) to B (**2**) was initially studied by feeding experiments carried out by Höfle and Kunze.[7] Importantly, they reported that the hydroxy group of aurachin B (**2**) at C3 is derived from molecular oxygen. Recent work by Pistorius et al.[8] discovered two gene loci containing aurachin biosynthetic genes in addition to the core biosynthetic gene cluster.[18–20] The authors speculated that two enzymes encoded by *auaG* and *auaH* are responsible for the migration of the farnesyl group from C3 to C4. However, the detailed biosynthetic conversion still remains to be solved, mainly because of the lack of detection of putative intermediates in vivo in *auaG* and *auaH* mutants of *S. aurantiaca* Sg a15.[8] To uncover the enzymatic chemistry behind this intriguing rearrangement reaction, we here describe in vitro experiments using recombinant AuaG and AuaH proteins.

AuaG belongs to the family of flavin-dependent monooxygenases and appears to be relatively similar to PgaE involved in angucycline biosynthesis according to results from BLAST search and Phyre2 analysis, respectively.[21] The

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protein possesses conserved motifs, GxGxxG, DG and GD, which are important for this class of enzymes.[22] AuaH seems to possess a Rossman fold and therefore was speculated to be a NAD(P)H-dependent reductase (NAD = nicotinamine–adenine dinucleotide). Both the conserved TGxxxGxG motif for NAD(P)H binding and the catalytic centers important for ketoreduction activity (YxxxK motif and the catalytic Ser residue) are present in AuaH.[23,24]

To analyze these enzymes in vitro, recombinant proteins fused with a hexahistidine tag at the C-terminus of AuaG and AuaH were expressed in *Escherichia coli* using the pET (Novagen) and pCold (Takara) systems, respectively. The recombinant proteins were purified using Ni^{2+} affinity chromatography (Supporting Information, Figure S1). AuaG was purified as a slightly yellow protein and the binding cofactor was confirmed to be flavin–adenine dinucleotide (FAD) by the method described previously (Figure 1),[25] thus

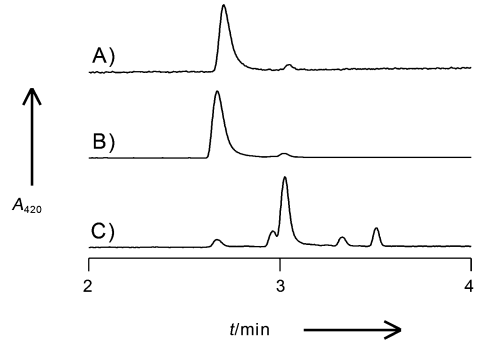


Figure 1. Analysis of the co-factor bound to AuaG. AuaG solution was mixed with equal volume of methanol and centrifuged to remove the precipitated protein. The obtained solution was analyzed by LC-MS analysis (A) and compared with authentic FAD (B) and flavin mononucleotide (FMN;C).

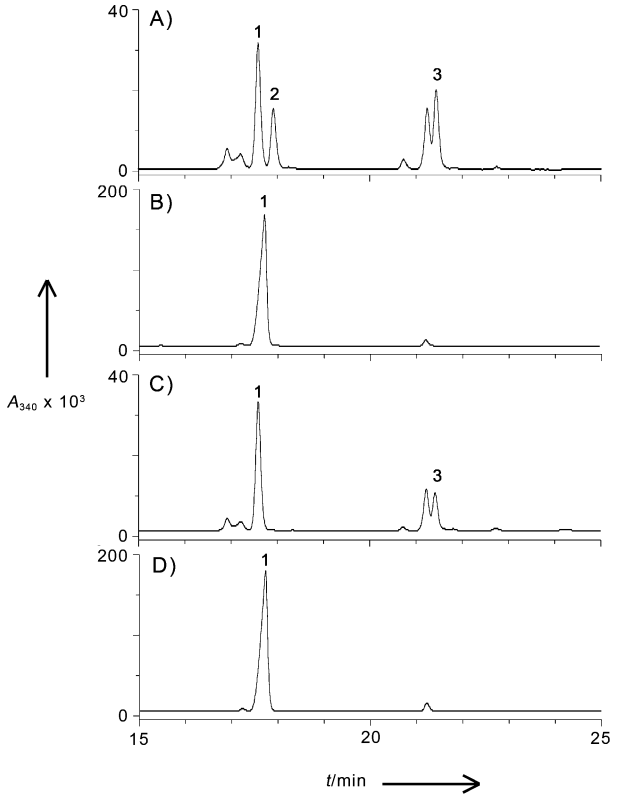


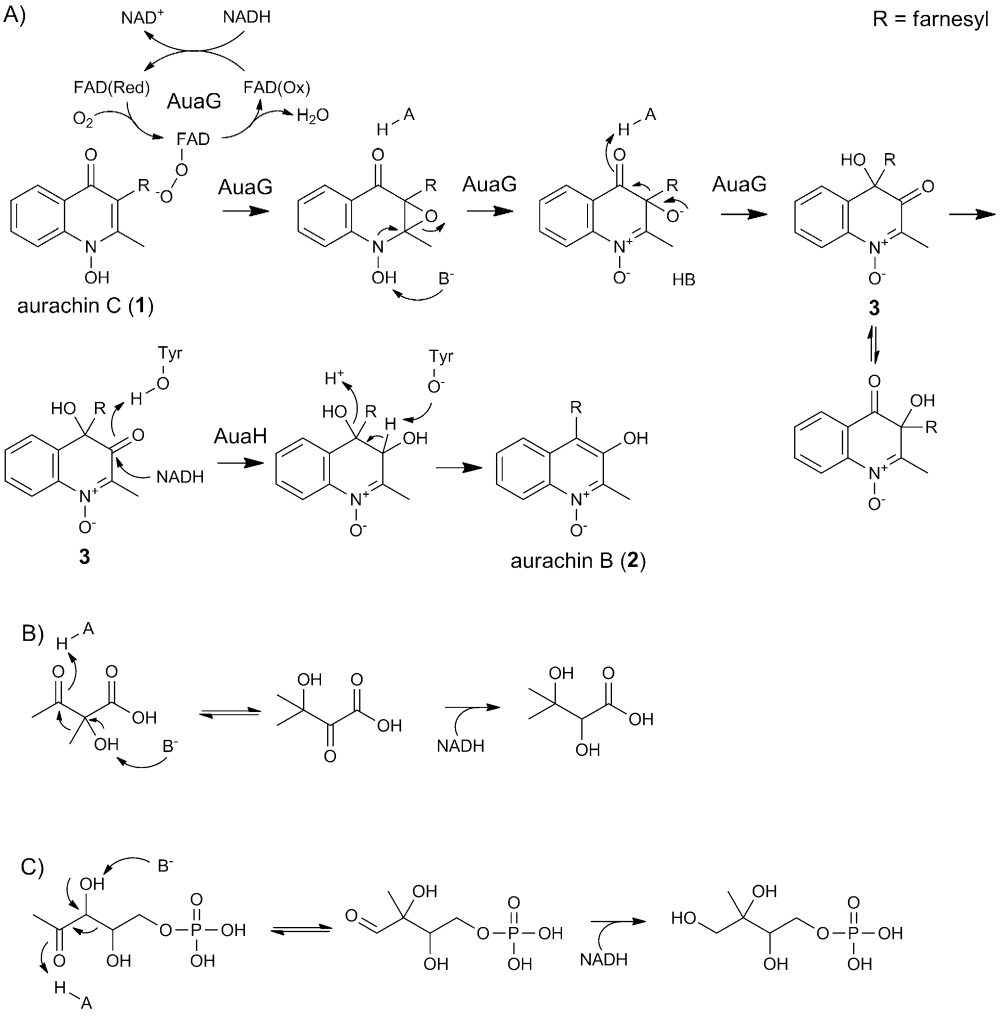
Figure 2. LC-MS analysis of the conversion of aurachin C (**1**) from AuaGH in vitro assays. When AuaG and AuaH were incubated with aurachin C (**1**) in the presence of NADH, synthesis of aurachin B (**2**) and oxidized aurachin C (**3**) was observed (A). When inactive AuaG and active AuaH was used, neither **3** nor aurachin B (**2**) was produced (B). When AuaG and inactive AuaH were used, only production of **3** was observed (C). In case of inactivation of both enzymes, no reaction took place (D).

indicating that approximately 80% of AuaG did bind FAD. When these recombinant enzymes (2 μM each) were incubated with 200 μM aurachin C (**1**) for 30 min at 30°C in a buffer containing 2 mM NADH and 0.1 mM of CHES (pH 9.5), the formation of aurachin B (**2**) was detected by LC-MS analysis (Figure 2A). The formation of aurachin B (**2**) was confirmed by comparison with an authentic sample with respect to retention time, UV, MS, and MS/MS spectra (Supporting Information, Figure S2). This experiment also revealed the production of unknown compounds. These exhibited a molecular weight that is 16 Da heavier than aurachin C (**3**) and were therefore speculated to be oxidized forms. When heat-inactivated AuaG was added to the reaction, neither aurachin B (**2**) nor oxidized aurachin C (**1**) was detected (Figure 2B). Once AuaH was heat inactivated, the formation of aurachin B (**2**) was eliminated but oxidized aurachin C (**3**) was still produced (Figure 2C). Furthermore, aurachin B (**2**) could also be formed by incubating AuaH with NADH plus an ethyl acetate extract of AuaG reaction products (Supporting Information, Figure S3). These results clearly show that AuaG and AuaH act sequentially and that conversion of aurachin C (**1**) into aurachin B (**2**) occurs by oxidation and subsequent reduction. Both enzymes required NADH or NADPH for the reaction, and they both showed a preference for NADH over NADPH (Supporting Information Tables S1, S2). Addition of FAD to the reaction mixture did not significantly influence the reaction velocity of AuaG. Based on the key amino acids that have been reported to constitute the catalytic center of ketoreductases associated with secondary metabolite biosynthetic pathways, S111A and Y139F mutants of AuaH were constructed. Both mutated enzymes failed to produce aurachin B (**2**) in vitro (Supporting Information, Figure S3).[23,24]

Compound **3** seemed to be the main product of AuaG. This compound was highly unstable and rapidly isomerized to a second compound in 0.1M CHES (pH 9.5)/methanol (Supporting Information, Figure S4). The resulting isomer displayed identical MS/MS fragments, suggesting that the two compounds have similar core structures (Supporting Information, Figure S2). However, it exhibited a UV spectrum different from compound **3**, probably because of a structural variation in the configuration of the conjugated double bonds (Supporting Information, Figure S2). Therefore we presume that the isomer is an oxidized form of aurachin C, the farnesyl side chain of which is found in a different position from compound **3**. Our efforts to stabilize the intermediate by

changing the pH from neutral to basic led to various ratios of compound **3**, isomers, and decomposition products (Supporting Information, Figures S4, S5). In equilibrium state, the ratio of isomer to compound **3** was approximately 9 at pH 9.5 and 1.4 at pH 7. Rapid degradation of compound **3** was observed under acidic conditions (Supporting Information, Figure S6).

To gain further insight into the reaction catalyzed by AuaGH we attempted to characterize the oxidized form of aurachin C (**3**), as it seemed to be the major product of the reaction catalyzed by AuaG. Unfortunately, isolation of compound **3** failed owing to its instability. Thus, we aimed to characterize the compound by direct analysis of the enzymatic reaction by NMR spectroscopy. The AuaG reaction mixture was extracted with dichloromethane and the obtained organic layer was buffered with potassium phosphate buffer (pH 7). Dichloromethane was evaporated under cold N_2-stream and the residual crude product was immediately dissolved in CD_3CN for NMR analysis. Prior to NMR analysis, the obtained sample was analyzed by LC-MS to confirm that compound **3** was the main product (Supporting Information, Figure S7). NMR analysis was performed by combination of 1D and 2D experiments in CD_3CN. Compound **3** was found much more stable in organic solvent than in water, although slight degradation of the compound after NMR analysis was still observed. Detailed analysis of the NMR data of the crude mixture revealed that the farnesyl group in the intermediate (**3**) has already rearranged from C3 to C4. Evidence for this was obtained from the HMBC spectra. HMBC correlations to a carbonyl group with a chemical shift of δ 195.3 could be detected for the methyl group at δ 2.23 (9-H) and a CH_2 group at δ 2.64 and 2.53(1′a,b-H) respectively. Furthermore, the signals of 5-H and 1′a,b-H showed HMBC correlations to a hydroxylated quartenary carbon at δ 77.5 (C4). These facts led us to conclude that the farnesyl group is attached to C4 in **3**, and **3** was thus identified as 4-hydroxy-2-methyl-3-oxo-4-farnesyl-3,4-dihydroquinoline-1-oxide.



Scheme 2. Proposed reaction mechanism of AuaG reaction (A) and comparison with the reaction of acetohydroxy acid isomeroreductase (B) and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (C), which are two enzymes that are known to catalyze the semipinacol rearrangement.

According to these results, we propose that AuaG catalyzes the migration of the farnesyl chain by a semipinacol rearrangement starting from aurachin C (Scheme 2).[9] First, AuaG epoxidizes the double bond at C2–C3 position of the quinoline core. After epoxidation, the *N*-hydroxy group is likely to be deprotonated in conjunction with activation of the carbonyl group by coordinative protonation. This type of acid–base catalysis may induce the ring opening of the epoxide followed by a semipinacol rearrangement, resulting in migration of the farnesyl group from position C3 to C4 to give rise to compound **3**. Such proton shuttling is also observed in other types of enzymes catalyzing semipinacol rearrangements, which are discussed below.

Both epoxidation and rearrangement are likely to be catalyzed by AuaG for the following reasons: First, the reaction seems to be selective for compound **3** under optimized conditions (Supporting Information, Figure S7). Second, compound **3** was rapidly converted into its isomer under basic conditions (Supporting Information, Figure S4). This result suggests that the intermediate **3** is not the favored state of the semipinacol rearrangement equilibrium and

therefore the reaction is likely to be driven enzymatically. The rearrangement appears to take place in an inverse direction under neutral and basic conditions, which is probably driven by the deprotonation of the hydroxy group at position C4, thus providing electrons to push back the farnesyl group to the C3 position. The forward rearrangement may be stimulated by a similar mechanism, but the "inverse isomer" is likely to be more favored because C3 has a stronger electrophilic property that is probably enhanced by the *N*-oxide at N-1. Strong support for this proposal comes from the description of 2-benzoyl-3-isonitrosobutan-2-ol, which was prepared under strong acidic conditions.[26] The equilibrium state described above and the instability of compound **3** suggested the requirement of immediate reduction catalyzed by AuaH to fix the farnesyl group at the position C4 and prevent the degradation of this intermediate.

To the best of our knowledge, there are only two examples of enzymes which catalyze semipinacol rearrangements (acyloin rearrangements):[12–17] Acetohydroxy acid isomeroreductase and 1-deoxy-D-xylulose-5-phosphate reductoisomerase are involved in valine/isoleucine biosynthesis[12–14] and 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, respectively.[15–17] (Scheme 2 B,C). Both enzymes exhibit dual functionality by catalyzing isomerization followed by reduction at a single active site and require divalent metal ions for isomerization in addition to NAD(P)H. As proposed by Tyagi et al., the reduction is important for overcoming the unfavorable equilibrium state of the isomerization. In contrast, AuaG does not require divalent metal ions and catalyzes isomerization coupled with epoxidation, not with reduction. AuaG probably establishes a selective isomerization by catalyzing a rearrangement coupled with the ring opening of an epoxide. The main product of AuaG appears to be an energetically less-favored isomer that is immediately stabilized by reduction and subsequent aromatization. The latter step is catalyzed by AuaH, which is thus required to complete the rearrangement.

In summary, we have reported a novel enzymatic system that establishes prenyl migration by employing a novel FAD-dependent monooxygenase (AuaG), which catalyzes epoxidation coupled with semipinacol rearrangement, and a ketoreductase (AuaH). This discovery provides important information regarding enzymatic rearrangements in natural product biosynthesis and triggers efforts to achieve biomimetic semipinacol rearrangements in organic synthesis.

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