A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

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Accepted Article

Title: Unveiling Two Consecutive Hydroxylations: Mechanism of Aromatic Hydroxylations Catalyzed by Flavin-Dependent Monooxygenases for Biosynthesis of Actinorhodin and Related Antibiotics

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To be cited as: *ChemBioChem* 10.1002/cbic.201900490

Link to VoR: http://dx.doi.org/10.1002/cbic.201900490

A Journal of ChemPubSoc Europe

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10.1002/cbic.201900490

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COMMUNICATION

Unveiling Two Consecutive Hydroxylations: Mechanism of Aromatic Hydroxylations Catalyzed by Flavin-Dependent Monooxygenases for Biosynthesis of Actinorhodin and Related Antibiotics

Makoto Hashimoto[†][a], Takaaki Taguchi[†][b], Kazuki Ishikawa[a], Ryuichiro Mori[a], Akari Hotta[a], Susumu Watari[a], Kazuaki Katakawa[a], Takuya Kumamoto[c], Susumu Okamoto[d] and Koji Ichinose*[a]

Abstract: Flavin-dependent monooxygenases are ubiquitous in living systems and are classified into single- or two-component systems. Actinorhodin produced by *Streptomyces coelicolor* is a representative polycyclic polyketide that is hydroxylated by the ActVA-5/ActVB two-component hydroxylase system. These homologous systems are widely distributed in bacteria, but their reaction mechanisms remain unclear. The present *in vitro* investigation provided chemical proof of two consecutive hydroxylations via hydroxynaphthalene intermediates involved in actinorhodin biosynthesis. The ActVA-5 oxygenase component catalyzed a stepwise dihydroxylation of the substrate, whereas the ActVB flavin reductase not only supplied a reduced cofactor, but also regulated the quinone-hydroquinone interconversion of an intermediate. Our study provides clues for understanding the general biosynthetic mechanism of highly functionalized aromatic natural products with structural diversity.

Flavin-dependent monooxygenases (FMOs) are distributed in animals, plants, and microorganisms, where they play essential roles in a variety of biological processes. Animal FMOs primarily oxygenate heteroatom-containing drugs and xenobiotics, producing metabolites such as *N*- and *S*-oxides that are readily excreted from the body.[1] The plant FMO YUCCA is a tryptamine *N*-hydroxylase for auxin biosynthesis,[2] whereas the yeast yFMO is involved in disulfide bond formation during protein folding through regulation of the glutathione/glutathione disulfide ratio.[3] A plethora of microbial FMOs have been reported to be involved in the degradation of environmental aromatic compounds, and in the biosynthesis of secondary metabolites.[4] These FMOs are classified into two types: single-component systems have both flavin reduction and oxygenation domains in a single polypeptide, whereas two-component systems comprise distinct flavin reductase and oxygenase proteins. Functional and structural studies of FMOs have mostly focused on the single-component systems. Consequently, few two-component systems have been characterized at the mechanistic level, particularly those for the biosynthesis of complex natural products.[5, 6]

Here, we focus on the pyranonaphthoquinone antibiotic actinorhodin (ACT, **1**) produced by *Streptomyces coelicolor* A3(2) as the sole known example of a polycyclic aromatic compound hydroxylated by a two-component FMO (Scheme 1). The FMO for ACT biosynthesis is the ActVA-ORF5/ActVB system consisting of an oxygenase, ActVA-5, and a flavin:NADH oxidoreductase, ActVB. Previously, we demonstrated *in vivo* that the system was a bifunctional oxygenase, governing quinone-formation at C-6 and hydroxylation at C-8 of a tricyclic ACT intermediate, 6-deoxy-dihydrokalafungin (DDHK, **2**) (Scheme 1).[7] Although **2** was deduced to be the natural substrate of the system,[7] it has never been isolated from any biosynthetic mutants or strains of *S. coelicolor* due to an oxidative dimerization leading to the formation of a shunt product, actinoperylone.[8] Thus, *in vitro* characterizations were limited by this fact, and emodinanthrone was used as a surrogate tricyclic substrate.[7]

The present study describes a semisynthetic preparation of **2** from (*S*)-DNPA (**3**), an isolable intermediate of ACT biosynthesis (Scheme 1), and the unambiguous chemical proof of the two consecutive hydroxylations of **2**, revealing the previously uncharacterized machinery for the interconversion between the quinone- and hydroquinone-form of intermediates governed by the ActVA-5/ActVB system. Furthermore, we demonstrated the monohydroxylation or dihydroxylation activity of the closest homologs of ActVA-5, Med-7 and Gra-21, for medermycin (MED, **4**) and granaticin (GRA, **5**) biosyntheses (Scheme 1). We propose that iterative oxidation catalyzed by two-component FMOs provides an explanation for the structural diversity in oxygen-containing natural products.

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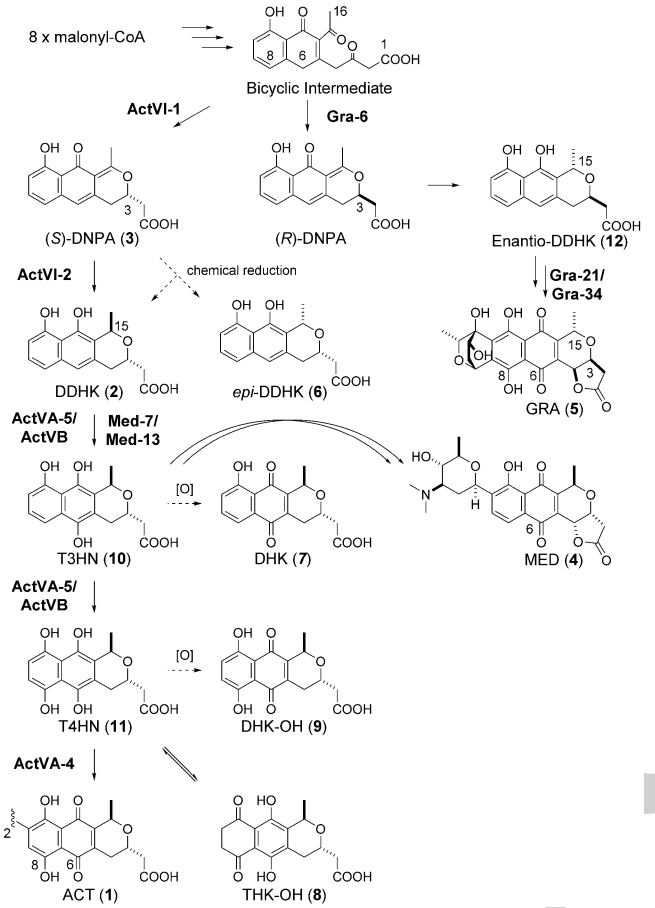
10.1002/cbic.201900490



Scheme 1. The proposed biosynthetic pathways of **1**, **4** and **5**. Position number is based on the polyketide (C16) biosynthetic origin. A common bicyclic intermediate[9] undergoes stereospecific keto-reductions at C-3/C-15 leading to either **2** or **12**. Two hydroxylations at C-6/C-8 and a single hydroxylation at C-6 are involved in ACT/GRA and MED biosynthesis, respectively.[10] ActVA-4 is a postulated enzyme involved in a dimerization step during ACT biosynthesis.[11] The dotted arrows depict chemical conversions of the related metabolites.

For preparation of substrate **2**, **3** [12] was reduced by $NaBH_4$, resulting in two compounds, which were assumed to be **2** or its C-15 epimer, *epi*-DDHK (**6**), from UV-VIS and MS spectra (RT 19 min and 20 min, Figure S1). Each was purified by preparative HPLC, and subjected to extensive NMR spectral analysis (Figure S1-S13, Table S1, S2). These spectra established that their planar structure is **2**. The NOESY spectrum of the compound with the earlier HPLC retention time (Figure S7) indicated an NOE correlation between 3-H and 16-H, proving its structure as **2** with the (3*S*, 15*R*) configurations. Likewise, the reasonable NOE correlation for the other compound (Figure S13) elucidated its absolute structure as **6**. Thus, we succeeded in establishing the semisynthetic method for preparing **2** together with **6** for the first time. Since unstable properties prone to decomposition of **2** were observed, it was stored at -30°C under a nitrogen atmosphere.

First, we optimized *in vitro* assay conditions for ActVA-5/ActVB based on our previous studies with model substrates.[7] To minimize an unwanted degradation of **2**, we employed both slightly acidic conditions (pH 6.5) and methanol as a solubilizing agent for **2**, followed by a mild heat treatment (see Supporting Information) for enzyme inactivation prior to HPLC analysis. The resultant assay workflow produced about 23% loss of **2** in the absence of ActVA-5/ActVB (Figure S14). After enzymatic reaction, we detected the peaks of hydroxy-tetrahydrokalafungin (THK-OH, **8**) and 8-hydroxy-dihydrokalafungin (DHK-OH, **9**), both of which were previously isolated from a deletion mutant of the *act*VA-ORF4 gene of *S. coelicolor* A3(2).[11] Their identical RT and UV-VIS spectra with the authentic ones were confirmed (Figure 1A, Figure S15), and notably, the conversion of **2** to **8** was almost quantitative (93 % yield). Without ActVA-5 or VB, unreacted **2** together with a small amount of **7** was detected (Figure 1B, 1C); **7** is the quinone form of a postulated C-6 oxygenated product, T3HN (**10**), possibly derived from autoxidation. Additionally, a series of control experiments were conducted to evaluate the requirement of each component in the assay mixture (Figure S16): 1) a higher amount of **2** was detected in the absence of enzymes; 2) the spontaneous formation of **7** was up to 10 % that of **2**; 3) NADH and FMN were essential for the activity; 4) the omission of catalase led to a reduction in activity. The combined results clearly showed that **2** is indeed the native substrate of the ActVA-5/ActVB system to conduct the two consecutive hydroxylations at C-6 and C-8.

When **7** was subjected to the assay, the formation of **8** was observed, suggesting that **7** is once reduced back to **10** by the ActVA-5/ActVB system (Figure 1D). The C-8 hydroxylation of **7** was not detected in our previous investigation,[7] where ethylene glycol monomethyl ether (EGME) was included in the assay mixture as a solubilizing agent. This result might have been due to the presence of hydrogen peroxide, an inevitable byproduct in the assay mixture, which is known to convert EGME to 2-methoxyacetoaldehyde, which might inhibit the conversion of **7** to **10**.[13]

The successful second hydroxylation of **7** at C-8 resulted in notable HPLC peaks **X** and **Y** at ca. 3 min (Figure 1D). Considering the co-presence of the quinone-form of the C-6/C-8 oxygenated products, **7** and **9**, we assumed **X** and **Y** to be the immediate enzymatic products, **10** and T4HN (**11**), respectively. The treatment of **7** solely with ActVB produced a substantial amount of **X** (3.2 min), whose formation was confirmed to be dependent on ActVB, NADH, and FMN (Figure 1E, S17). The similarity in UV-VIS spectra of **X** and **2** also suggested their naphthalene chromophores (Figure S18). LC/HRESIMS analysis of the reaction mixture containing **X** revealed a molecular formula of $C_{16}H_{16}O_6$ exactly matching that of **10** (Figure S18). An earlier study showed that sodium dithionite ($Na_2S_2O_4$) converts an anthraquinone to the corresponding anthrahydroquinone[14], which led us to treat **7** with $Na_2S_2O_4$ to demonstrate the chemical formation of **X** (Figure S18). In fact, peak **X** was present in the HPLC analysis of the standard assay mixture (Figure 1A), most likely to be undergoing the second hydroxylation. All the results strongly suggest **X** to be **10**, itself.

Similar ActVB-dependent formation of **Y** (3.3 min) from **9** was observed (Figure 1F). Its rigorous LC/HRESIMS analysis resulted in the MS detection corresponding to a molecule of $C_{16}H_{16}O_8$ (Figure S20)**,** which is an oxygenated formula of **11** or **8**. Although $Na_2S_2O_4$ treatment readily converted **9** to **8**, the reduction in the amount of $Na_2S_2O_4$ selectively produced **Y** instead of **8** (Figure S

21). In fact, **Y** was detectable under the standard assay conditions (Figure 1A, 1D), indicating that **Y** could be an oxygenated shunt product derived from **11** or **8**.

The conversion by ActVB from the quinone (**7** and **9**) to hydroquinone (**10** and **11**) form of substrates is an additional important observation in this study. This *in vitro* quinone-reduction activity of ActVB was previously reported under anaerobic conditions[15] and it is now extended to aerobic conditions, possibly reflecting the significance of a separate reductase for an adequate supply of both reduced flavin cofactors and substrates.

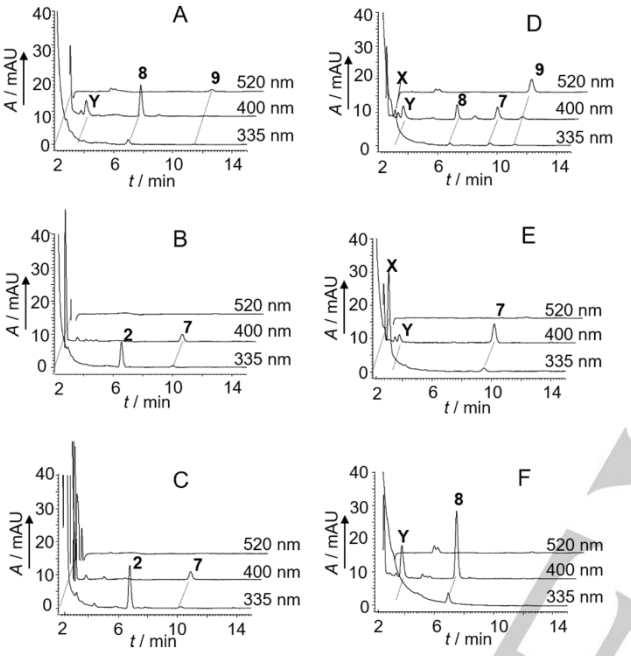


Figure 1. HPLC chromatograms of the reaction mixtures of various substrates with combinations of ActVA-5 and ActVB: (A) **2** with ActVA-5/ActVB; (B) **2** with ActVB; (C) **2** with ActVA-5; (D) **7** with ActVA-5/ActVB; (E) **7** with ActVB; (F); **9** with ActVB. See Supporting Information for details of the reaction conditions.

The mechanism of two consecutive hydroxylations is of great interest. Two mechanisms could be assumed: 1) once ActVA-5 accepts **2**, it catalyzes two hydroxylations at C-6 and C-8 consecutively in the active site(s) and releases the product **11** (single substrate recognition), or 2) ActVA-5 catalyzes the first hydroxylation at C-6 and releases the product **10**, which was 8-hydroxylated by a separate ActVA-5 (stepwise substrate recognition). In both cases, two molecules of C(4a)-FMN-hydroperoxide species ($FlHOO^-$)[16] are required in the reaction stoichiometry. The C_2 component of 4-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii* shows 44% similarity to ActVA-5 and its X-ray crystallographic study indicated that the C_2 protein accommodates a single molecule of FMN in its catalytic cavity.[17] Modeling studies on the ActVA-5 protein using C_2 protein (PDB ID: 2JBT) as a template (Figure S22, S23) allowed us to propose that the protein cavity would accommodate a single molecule of FMN.

Time-course analysis of **2**, **7**, and **8** under the standard assay condition revealed that **2** rapidly decreased within 1 min, and **8** was generated at 63% yield., while almost no **7** was detected (Figure 2A). Subsequent analysis with a reduced amount of ActVA-5 over 20 min revealed the accumulation of **7** in association with the decrease in **2**, followed by the significant generation of **8** (Figure 2B), favoring the step-wise recognition by the ActVA-5. Our earlier study[11] on an *act*VA-ORF4 deletion mutant (Scheme 1) suggested its involvement in the dimerization step of the ACT biosynthesis. This is based on the accumulation of **9** as well as **7** in the mutant, being in agreement with the step-wise hydroxylation steps proposed in this study.

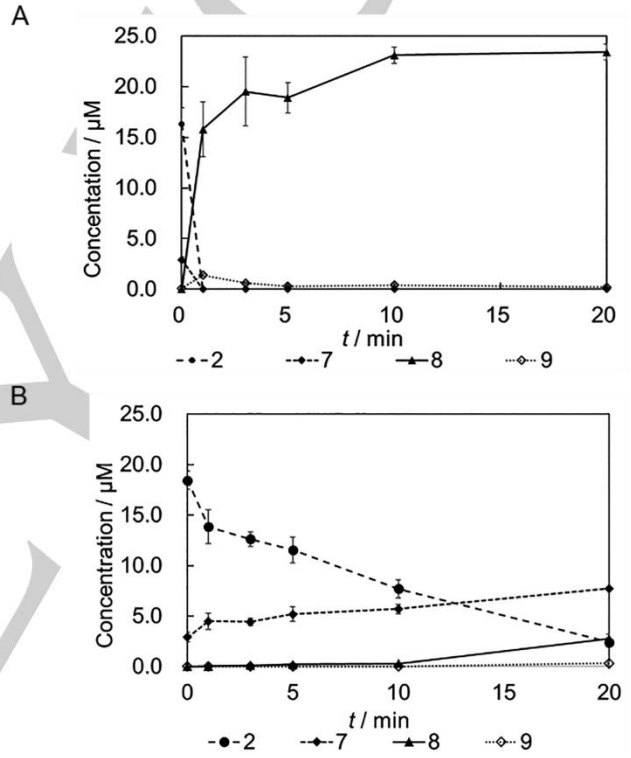


Figure 2. Time-course analysis of the ActVA-5/ActVB system: (A) the standard assay; (B) the assay with a reduced concentration (one-fifth) of ActVA-5 (means ±S. E., n = 3)

Our previous *in vivo* analysis showed that two ActVA-5 homologs, Med-7 and Gra-21 for the biosynthesis of **4** and **5** (Scheme 1), also have DDHK hydroxylation activity.[7] Their *in vitro* activities were also investigated using **2** and ActVB as a flavin reductase (Figure 3). The conversion yields of **2** to **8** and **9** of ActVA-5 and Gra-21 were 99% and 80%, respectively. Moreover, Gra-21 was confirmed to be a bifunctional FMO for C-6 and C-8 hydroxylations. Interestingly, the native substrate of Gra-21 should be enantio-DDHK (**12**) with (3*R*, 15*S*) configurations (Scheme 1). The stereochemical centers at C-3 and C-15 exerted no major influence on the C-6 and C-8 hydroxylation activities of Gra-21.

Med-7 has been suggested to be a monofunctional FMO.[7] Indeed, 7 was the main product of the *in vitro* hydroxylation

reaction, and the conversion yield of **2** to **7** was 45%. Interestingly, a small amount of **8**, equivalent to 6% of the substrate, was detected. These observations lead to the hypothesis that the affinity of Med-7 for **10** could be lower than those of ActVA-5 and Gra-21. Thus, Med-7 releases **10** immediately after C-6 hydroxylation, and the second hydroxylation reaction does not take place.

These functional differences between ActVA-5 and Med-7 should also be derived from the distinct structural determinants of substrate recognition. Modeling studies were extended to Gra-21 and Med-7 (Figure S22, 23), followed by the docking simulation of **2** and **10.** A marked difference was observed for the residues in the vicinity of ligand pockets (Figure S24). The sets of two aligned residues (^{227}R and ^{314}R/ActVA-5; ^{255}K and ^{336}R/Gra-21; ^{227}K and ^{313}R/Med-7) are particularly of interest. The two arginine residues of ActVA-5 (Figure S25), which function as a proton-donor to an anionic form of **2**, tightly accommodate a substrate. The same role would be undertaken by the single residue: ^{255}K/Gra-21 (Figure S26) and ^{358}S/Med-7 (Figure S27). It is noteworthy that ^{358}S/Med-7 is distantly positioned from ^{227}R/ActVA-5 and ^{255}K/Gra-21 in both the alignment and the models of the three related proteins (Figure S24). The Med-7 structure seems to form an apparently looser catalytic pocket for **2** (Figure S27) and **10** (Figure S28) than those of ActVA-5 (Figure S25) and Gra-21 (Figure S26). This difference could be explained by the lack of pi-stacking (^{234}F/ActVA-5, ^{262}F/Gra-21, ^{234}L/Med-7) and hydrophobic (^{122}M/ActVA-5, ^{147}M/Gra-21, ^{120}A/Med-7) interactions (Figure S25-S27) with a ligand molecule, which would contribute to the difference in turnover of a substrate and/or FNM, accounting for the mono-functionality of Med-7.

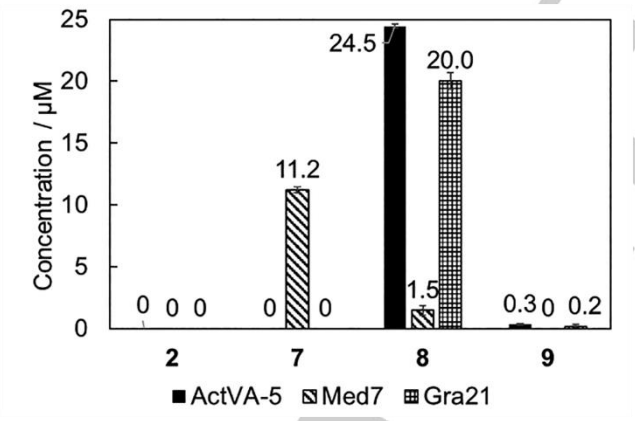


Figure 3. THK-OH production activities of ActVA-5, Med-7, and Gra-21 from **2** with ActVB as a FMN reductase (means ±S. E., n = 3)

We successfully established a semisynthetic procedure for preparing **2** and **6** from a biosynthetic intermediate **3** and an assay condition to achieve two consecutive hydroxylations at C-6 and C-8 of **2** to produce **8**. The bifunctionality of ActVA-5 indicated by *in vivo* analysis[7] was unambiguously proved by *in vitro* analysis. Time-course analysis suggests that the stepwise recognition mechanism would be supported. The fact that **2** is converted to **8** by ActVA-5/ActVB supports our previous proposal[11] that **11** would be a substrate for dimerization by regiospecific aryl-coupling, the last step in ACT biosynthesis. Recently, we established an *in vitro* reconstitution system using recombinant ACT biosynthetic enzymes to produce **3**.[9] In *S. coelicolor*, an enoylreductase encoded by *act*VI-ORF2 converts **3** stereospecifically to **2** (Scheme 1). [7, 8] Our ongoing studies are dealing with the functional expression of ActVI-2 to connect the earlier pathway to the present ActVA-5/ActVB system to reconstitute at least 25 steps from malonyl-CoA, covering the entire ACT biosynthetic pathway except for dimerization.

Two-component FMOs homologous to the ActVA-5/ActVB system are widely distributed in bacteria (Figure S29, Table S3, S4). This work demonstrated that a flavin reductase, ActVB, apparently regulates the interconversion between the naphthoquinone and hydronaphthoquinone form of ACT biosynthetic intermediates. This observation agrees with the recent finding of the importance of hydronaphthoquinone in biosynthesis,[18] and our results would provide an example of the general biosynthetic mechanism of highly functionalized aromatic natural products such as polyphenols and polymeric aryl compounds.

In conclusion, the ActVA-5/ActVB system provides a previously uncharacterized example of the hydroxylation of a polycyclic aromatic compound catalyzed by a two-component FMO.

Acknowledgements

We thank Mr. Y. Kanai, Mr. K. Maru, and Ms. E. Hoshi, Faculty of Pharmacy, Musashino University for their assitance in experiments. We also thank Mr. Yoshirou Kimura, Molsis Inc. for helpful discussion. This work was supprted by JSPS KAKENHI Grant Number JP17K08350 (to TT) and JP19H03385 (to KI).

Cnflict of Interest

The authors declare no conflict of interest.

Keywords: aromatic hydroxylation• biosynthesis• flavin-dependent monooxygenase• actinorhodin • reaction mechanisms

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10.1002/cbic.201900490

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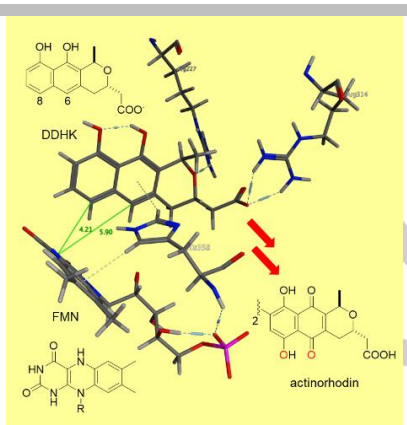
Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

Filling the gap: 6-Deoxy-dihydrokalafungin (DDHK) is a previously uncharacterized intermediate for actinorhodin (ACT) biosynthesis. Semisynthetic preparation of DDHK was established, followed by its use for in vitro enzymatic reactions using a flavin-dependent monooxygenase, ActVA-ORF5, and a flavin reductase, ActVB. DDHK was hydroxylated stepwise at C-6 and C-8, proving its ACT biosynthetic intermediacy.



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Unveiling Two Consecutive hydroxylations: Mechanism for Aromatic Hydroxylations Catalyzed by Flavin-Dependent Monooxygenases for Biosynthesis of Actinorhodin and Related Antibiotics