# Structure of the Dioxygenase AsqJ: Mechanistic Insights into a One-Pot Multistep Quinolone Antibiotic Biosynthesis

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Abstract: *Multienzymatic cascades are responsible for the biosynthesis of natural products and represent a source of inspiration for synthetic chemists. The Fe^{II}/α-ketoglutarate-dependent dioxygenase AsqJ from Aspergillus nidulans is outstanding because it stereoselectively catalyzes both a ferryl-induced desaturation reaction and epoxidation on a benzodiazepinedione. Interestingly, the enzymatically formed spiro epoxide spring-loads the 6,7-bicyclic skeleton for non-enzymatic rearrangement into the 6,6-bicyclic scaffold of the quinolone alkaloid 4′-methoxyviridicatin. Herein, we report different crystal structures of the protein in the absence and presence of synthesized substrates, surrogates, and intermediates that mimic the various stages of the reaction cycle of this exceptional dioxygenase.*

Dioxygen-activating non-heme Fe^{II}/α-ketoglutarate-dependent oxygenases participate in a vast array of biologically important and energetically demanding reactions, such as the stereoselective desaturation of unactivated carbon–carbon single bonds, oxidative ring closure, olefin epoxidation, hydroxylation processes, and the oxidation of alcohols, sulfides, and phosphines (Scheme 1a).[1] The substrate oxidation mediated by those enzymes is coupled to the decomposition of α-ketoglutarate (αKG) into CO_2 and succinate and likely involves a high-valent Fe^{IV}-oxo species as the key intermediate.[2] Recently, Watanabe and co-workers discovered the enzyme AsqJ from *Aspergillus nidulans* as a major constituent in the biosynthesis of 4′-methoxyviridicatin (**4**, Scheme 1b).[3] The characteristic 4-arylquinolin-2(1H)-one structure of **4** is found in a variety of quinolone alkaloids,[4] which are privileged molecules with antibacterial, antimalarial, antiviral, and antitumor activities.[5] A peculiar aspect of AsqJ is that it performs two distinct, stepwise oxidations on the AsqK-catalyzed nonribosomal peptide synthetase (NRPS) product 4′-methoxycyclopeptin (**1**, Scheme 1b): a double bond is created by desaturation to 4′-methoxydehydrocyclopeptin (**2**), which is then epoxidized to 4′-methoxycyclopenin (**3**; Figure 1). The last step triggers a fascinating
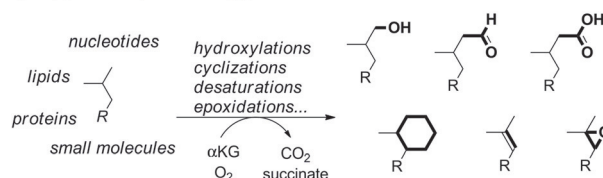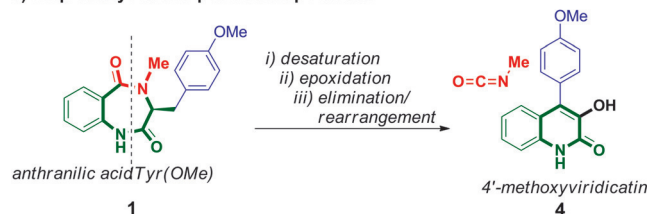
Scheme 1. a) The reactivity spectrum of Fe^{II}/αKG-dependent dioxygenases.[1] b) AsqJ converts 4′-methoxycyclopeptin (**1**) into the quinolone alkaloid 4′-methoxyviridicatin (**4**). The enzymatic desaturation and epoxidation steps are followed by a non-enzymatic rearrangement with concerted methylisocyanate (red) elimination. Substrate **1** is the AsqK-produced NRPS product of anthranilic acid and Tyr(OMe); the benzodiazepinedione N-methyl group is derived from S-adenosylmethionine.[9]

non-enzymatic elimination/rearrangement of the 6,7-bicyclic skeleton of **3** to the 6,6-bicyclic quinolone framework of **4**.[6] In order to understand this AsqJ-mediated reaction sequence of desaturation, epoxidation, and elimination/rearrangement, we aimed to obtain detailed structural information.

We cloned and expressed the *asqJ* gene from *A. nidulans* in *Escherichia coli* strain BL21(DE3). The recombinant dioxygenase was purified by Ni^{2+} affinity and size-exclusion chromatography. Subsequently, AsqJ was crystallized and its structure was determined through molecular replacement to a resolution of 1.7 Å ($R_{\text{free}} = 19.5\%$; PDB ID: 5DAP, see the Supporting Information) by using the coordinates of the phytanoyl coenzyme A hydroxylase EasH (PDB ID: 4NAO) as a starting model.[7] The fold of the homodimeric AsqJ protein displays striking similarities to that of EasH (C$_\alpha$ root-mean-square deviation (rmsd) 1.2 Å for 80% of C$_\alpha$ atoms; Figure S5 in the Supporting Information). The enzyme forms a funnel-like reaction chamber similar to the interior of the common jelly-roll barrel (Figure S1 in the Supporting Information).[8]

The substructure of AsqJ responsible for metal and αKG binding shows the characteristic H$_1$-X-D/E-X$_n$-H$_2$ motif of Fe^{II}/αKG dioxygenases, with the three metal-binding amino acids H134, D136, and H211 (Figure 2b).[1a] The octahedral coordination sphere of the metal in the resting state of the

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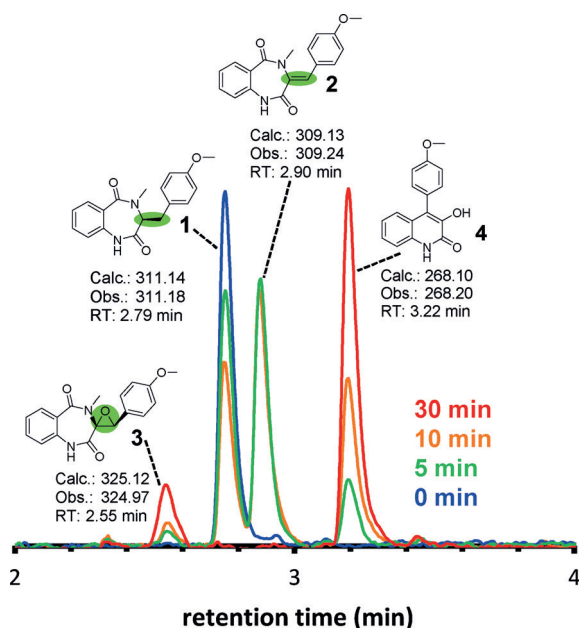Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/anie.201507835.

Figure 1. Reversed-phase HPLC/MS-coupled activity assay for AsqJ in the presence of Fe^{2+}, αKG, oxygen, and ascorbic acid. Shown is a chromatogram with absorption peaks ($\lambda = 280$ nm) for substrate **1**, two reaction intermediates **2** and **3**, and the final product **4**. The reaction progress was analyzed after 0 min (blue), 5 min (green), 10 min (orange), and 30 min (red), respectively.

enzyme is completed by the C-1 carboxylate and C-2 keto group of the cosubstrate αKG, and by a defined water molecule. Surprisingly, an X-ray fluorescence spectrum of the AsqJ crystals recorded at the synchrotron revealed the presence of nickel as the only heavy-metal atom. An anomalous dataset at the Ni edge ($\lambda = 1.4843$ Å) confirmed that this ion occupies the active site of the dioxygenase, from which it likely replaced the catalytic iron during the Ni^{2+}-affinity chromatography step.

To ascertain the activity of the enzyme, we exposed AsqJ to its substrate 4′-methoxycyclopeptin (**1**), which was synthesized in one step from isatoic anhydride and *N*-methyl-Tyr(OMe) (see the Supporting Information).[10] The addition of 100 μM Fe^{2+} (leading to Ni^{2+}-to-Fe^{2+} exchange) was a prerequisite for restoring the activity of AsqJ (Table S1 in the Supporting Information). The enzymatically induced conversion of **1** into **4** was analyzed by an HPLC/MS-coupled activity assay and two reaction intermediates, the desaturase product **2** and oxirane **3**, were identified in addition to the final quinololone alkaloid **4** (Figure 1), which is in agreement with previous results.[3]

The Fe^{2+}-to-Ni^{2+} substitution resulted in enzyme inactivation and allowed us to determine the AsqJ structure with complexed substrate **1** through cocrystallization experiments.[11] The overall structure of the enzyme and the octahedral metal coordination geometry were unchanged by ligand binding. Compound **1** is located opposite to the H134-D136-H211 triad and is held by hydrogen bonds between the diazepinedione oxygen atoms and M137NH and N70N$^{\delta 2}$. The 7-membered ring of **1** is fixed in a boat conformation with a pseudo-equatorial methoxybenzyl substituent. This confor-
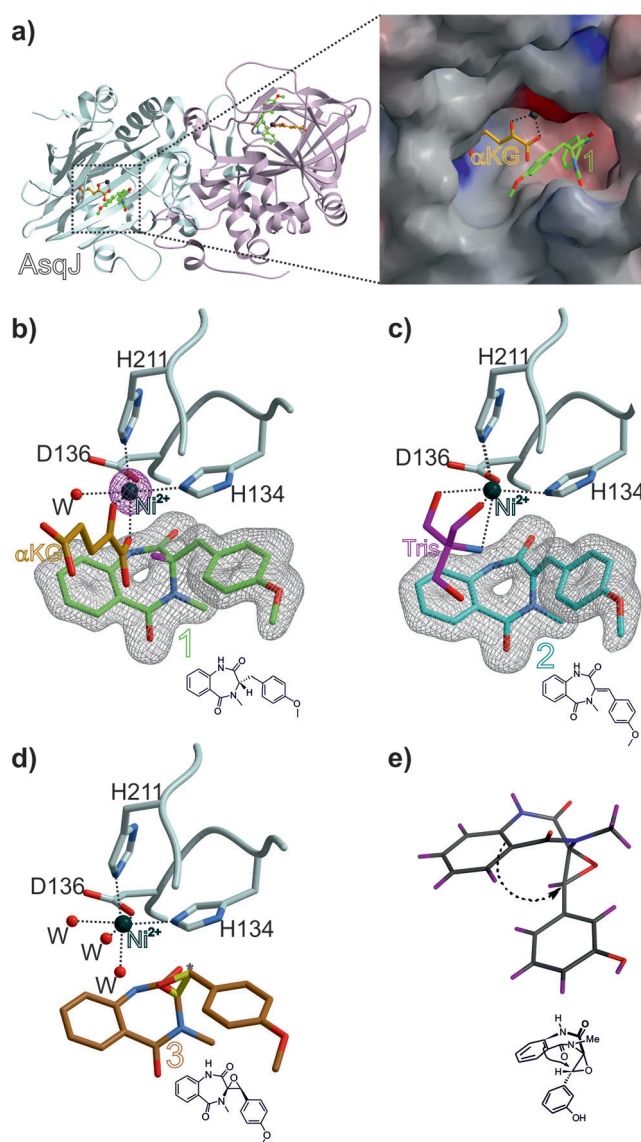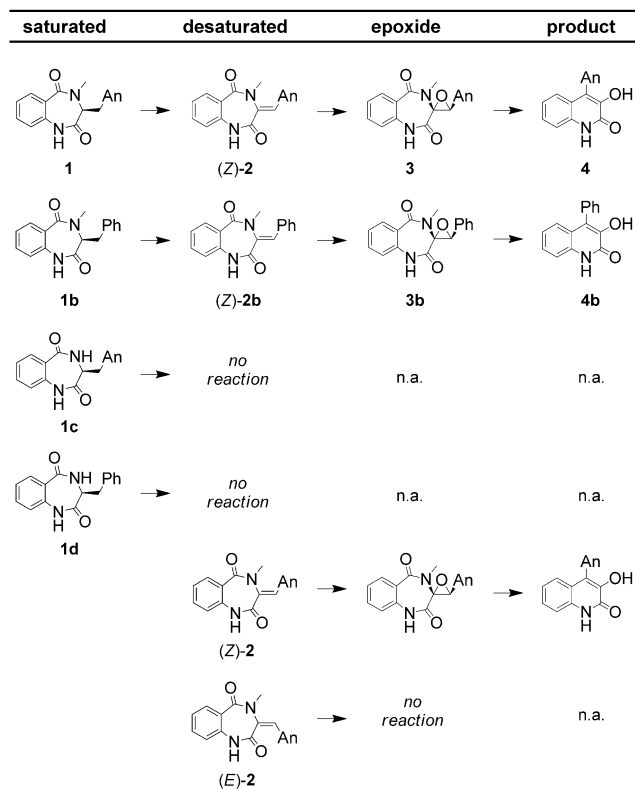

Figure 2. a) Ribbon diagram of the AsqJ homodimer with the enlarged active site shown as a potential surface contoured from $-30kTe^{-1}$ (red) to $+30kTe^{-1}$ (blue). The co-substrate α-ketoglaurate (αKG, gold) and the substrate **1** (green) are shown as stick models (PDB ID: 5DAQ). b) Structure of the AsqJ:**1** complex, showing the octahedral coordination of Ni^{2+} by the HxDx$_n$H motif, the C-2 oxo group and C-1 carboxylate of αKG, the H-3 hydrogen atom (magenta), and a water molecule (W). The 2Fo−Fc electron density map is contoured at 1σ (grey mesh), whereas the anomalous electron density (magenta) for Ni^{2+} is contoured at 10σ. c,d) AsqJ in complex with the desaturated intermediate **2** (PDB ID: 5DAV; notably, a Tris molecule coordinates the Ni^{2+} rather than the expected αKG), and the modelled epoxide **3**. e) The X-ray structure of an analogue of **3** with a 3-hydroxy group instead of the 4-methoxy group (CSD reference code POHBEV)[15] demonstrates the crucial preorientation of the spiro oxirane benzodiazepinedione system to induce the non-enzymatic elimination/rearrangement to yield product **4**.

mation corresponds to the less abundant of the two conformers of **1** observed in [D$_6$]DMSO solution. In the binding pocket, the substrate is perfectly orientated for a π-stacking interaction between its 4-methoxyphenyl (*p*-anisyl) group and
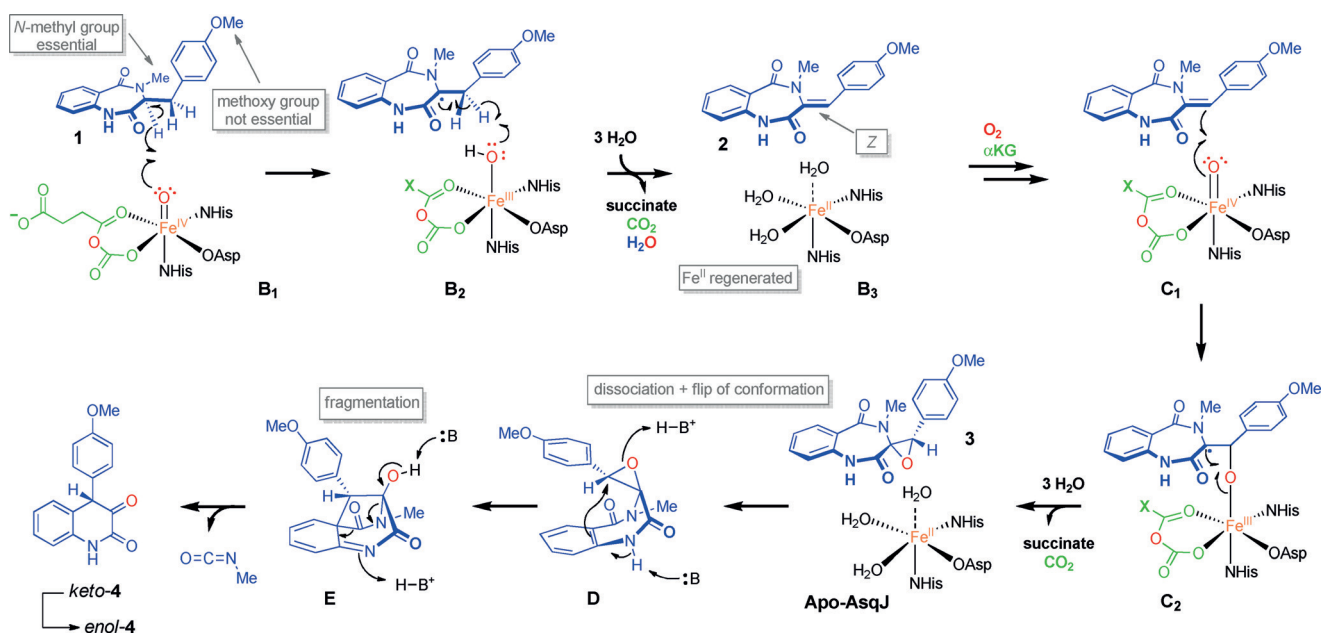
H134, with an interplanar distance of 3.6 Å and a displacement of 1.7 Å.[12] Both aryl rings of the substrate are stabilized by Van-der-Waals interactions with residues M118, L79, V72, and F139. The Ni^{2+} ion is at a distance of 4.5 Å to the designated C−C single bond to be desaturated. In line with the general mechanism suggested for Fe^{II}/αKG-dependent dioxygenases (Scheme S1),[1a,13] the next step is O$_2$ coordination to the iron center of the enzyme–substrate complex,[1a,14] with displacement of the defined water molecule from the sixth coordination position. Nucleophilic attack of the peroxo ligand at the keto carbonyl group of αKG forms a bicyclic Fe^{III} peroxyhemiketal complex (Scheme S1, **A**) that rearrange to the key Fe^{III}-hydroxyl radical or Fe^{IV}-oxo species (Scheme 3, **B$_1$**).[1a,13] The highly reactive Fe^{IV}-oxo species is poised to abstract a hydrogen atom from either C-3 or the benzylic C-1′ in **1** (Scheme 3, **B$_1$**) to generate a substrate radical in the vicinity of the hydroxo-Fe^{III} intermediate.[1a] Next, the second hydrogen atom is abstracted to stereoselectively produce alkene (Z)-**2** (Scheme 3, **B$_2$**–**B$_3$**). Decomposition of the αKG-derived co-ligand with release of carbon dioxide and succinate regenerates the active center in its Fe^{II} form.

Next, we performed in vitro activity assays of AsqJ with the synthesized substrate analogues **1b**–**d** (Scheme 2). Together with the crystallographic results, this study allowed us to explore the minimal requirements for ligand binding and conversion. A surrogate lacking the methoxy group (**1b**) was still converted by the enzyme via the reaction sequence of desaturation, epoxidation, and elimination/rearrangement, which contrasts with previous observations (Scheme 2, Figure S8).[3] Interestingly, methylation at N-4 is of major importance for catalysis since no turnover was detected with analogues **1c** and **1d**, which both lack the benzodiazepine-dione N-methyl group (Scheme 2). This finding is in line with the presence of an N-methylation domain in the NRPS AsqK.

However, the AsqJ:**1d** cocrystal structure (1.7 Å resolution, $R_{free} = 19.4$; PDB ID: 5DAX) revealed that the surrogate **1d** adopts a conformation identical to that of substrate **1** in the AsqJ:**1** complex. We thus conclude that the N-4 methylation

saturated	desaturated	epoxide	product
1	(Z)-**2**	**3**	**4**
1b	(Z)-**2b**	**3b**	**4b**
1c	*no reaction*	n.a.	n.a.
1d	*no reaction*	n.a.	n.a.
(Z)-**2**			
(E)-**2**	*no reaction*	n.a.	

Scheme 2. Substrate analysis study for the AsqJ-catalyzed reaction. An = p-anisyl (p-methoxyphenyl), n.a. = not available. Also see Table S1.

Scheme 3. Proposed mechanism for the AsqJ-mediated conversion of 4′-methoxycyclopeptin (**1**) into 4′-methoxyviridicatin (**4**). The rearrangement of **3** into **4** is enzyme independent.

likely exerts an electronic effect but is not required for the substrate fit.

Since AsqJ has the capability to catalyze both desaturation and epoxidation reactions, we aimed to elucidate the relationship between the two oxidative processes. Intermediate **2** was synthesized by condensing isatoic anhydride and sarcosine to give a benzodiazepinedione core,[10] to which an exocyclic 4-anisylidene group was subsequently attached in a Perkin-like condensation with 4-methoxybenzaldehyde (see the Supporting Information).[16] Starting the AsqJ-catalyzed reaction from **2** indeed led to the generation of both oxirane intermediate **3** and product **4**, but only in the presence of Fe^{2+}, αKG, and oxygen. These findings demonstrate that the desaturation and epoxidation are decoupled operations, with each of them requiring one molecule of αKG, as well as oxygen. The regenerated Fe^{II} center (Scheme 3, Apo-AsqJ) is thus ready to initiate the epoxidation once the succinate from the dehydrogenation step has been replaced by a new αKG co-substrate. Notably, AsqJ only showed activity with *Z*-configured **2**, but not towards its geometrical *E* isomer **2′** (Scheme 2, and Figure S7 in the Supporting Information). Accordingly, we obtained an AsqJ complex structure solely with **2** (Figure 2c, PDB ID: 5DAV), thus demonstrating that the *Z* isomer of product **2** but not the *E* isomer **2′** is able to bind into the active site cavity.

In the second Fe^{II}/α-ketoglutarate reaction cycle, the ferryl Fe^{IV}-oxo species converts the desaturated intermediate **2** into epoxide **3** (Scheme 3, **C1** and **C2**). Although a crystal structure of the AsqJ complex was not achievable, it is most likely that the enzyme-bound oxirane **3** adopts an identical conformation to that observed with **1** and **2**, since the protein environment only tolerates a single extended boat configuration (Figure 2d). Importantly, this mandatory substrate specification imposed by AsqJ is in direct contrast to the "small-molecule" X-ray structure of 3′-hydroxycyclopenine (CSD reference code POHBEV).[15] The three-dimensional structure of the close analogue of **3** displays the molecule in a substantially different flipped conformation, which allows an energetically favorable intramolecular π–π interaction between the two aromatic ring systems (Figure 2e and Figure S3b in the Supporting Information). However, it is this flipped spatial arrangement of **3** that is fundamental to initiate the nucleophilic attack of the *ortho* carbon atom of the arylamide moiety on the benzylic oxirane carbon atom (Scheme 3 , **D**). As a result, the AsqJ-bound intermediate **3** is forced into a non-reactive extended conformation (Figure S4). Consequently, the final elimination and rearrangement of **3** takes place non-enzymatically by a spontaneous process that occurs after dissociation from the dioxygenase. In this reaction, the tricyclic core of the presumed intermediate **E** fragments with elimination of methylisocyanate to yield the keto form of **4**, which eventually tautomerizes to its aromatic enol form.[6,17] In essence, the enzymatically formed spiro epoxide spring-loads the 6,7-bicyclic skeleton for rearrangement into the 6,6-bicyclic quinolone framework of 4′-methoxyviridicatin **4** as a result of ring strain and proper structural preorganization.

In summary, the biosynthesis of 4-arylquinolin-2(1*H*)-one alkaloids involves an intricate sequence of dehydrogenation and epoxidation, with a surprising final fragmentation under release of methyl isocyanate leading to the target heterocyclic structures. Notably, it has been reported that three enzymes are involved in an analogous benzodiazepine-quinolinone pathway for the biosynthesis of viridicatin in *Penicillium cyclopium* or *P. viridicatum*: a dehydrogenase, an epoxidase, and an enzyme called cyclopenase, which is responsible for the rearrangement.[18] AsqJ is so far unique in performing the elucidated desaturation, epoxidation, and elimination/rearrangement sequence. The only other dioxygenase that catalyzes a sequential dehydrogenation–epoxidation reaction is involved in pentalenolactone sesquiterpenoid biosynthesis in *Streptomyces*.[19]

The specific conformational control over reaction selectivity displayed by this system is a peculiarity of enzymatic reactions and not typically attained or imitated by metal-complex model systems. AsqJ is a catalyst that induces a remarkable one-pot multistep synthesis accompanied by major structural changes and it thus represents a particularly efficient type of synthetic reagent.

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