Natural Products

DOI: 10.1002/anie.201407920

Non-Heme Dioxygenase Catalyzes Atypical Oxidations of 6,7-Bicyclic Systems To Form the 6,6-Quinolone Core of Viridicatin-Type Fungal Alkaloids**

*Noriyasu Ishikawa, Hidenori Tanaka, Fumi Koyama, Hiroshi Noguchi, Clay C. C. Wang, Kinya Hotta, and Kenji Watanabe**

Abstract: *The 6,6-quinolone scaffold of the viridicatin-type of fungal alkaloids are found in various quinolone alkaloids which often exhibit useful biological activities. Thus, it is of interest to identify viridicatin-forming enzymes and understand how such alkaloids are biosynthesized. Here an Aspergillal gene cluster responsible for the biosynthesis of 4′-methoxyviridicatin was identified. Detailed in vitro studies led to the discovery of the dioxygenase AsqJ which performs two distinct oxidations: first desaturation to form a double bond and then monooxygenation of the double bond to install an epoxide. Interestingly, the epoxidation promotes non-enzymatic rearrangement of the 6,7-bicyclic core of 4′-methoxycyclopenin into the 6,6-quinolone viridicatin scaffold to yield 4′-methoxyviridicatin. The finding provides new insight into the biosynthesis of the viridicatin scaffold and suggests dioxygenase as a potential tool for 6,6-quinolone synthesis by epoxidation of benzodiazepinediones.*

Quinoline and quinolone alkaloids are found in various organisms and display a broad spectrum of useful biological activities, including antibacterial, antimalarial, antiviral, and antitumor activities.[1] As such, the quinolone motif has served an important role as a flexible framework for preparing libraries of bioactive compounds.[2] 4′-Methoxyviridicatin (**1**), which is characterized in this study for the first time, and related viridicatin (**2**), which have been isolated from various *Penicillium* sp.,[3] contain a structurally interesting and pharmaceutically attractive scaffolds[4] (Figure 1 A). This scaffold is found in various quinolone and quinolinone alkaloids.[5] The viridicatin framework is thought to be formed from benzodiazepinediones,[4,6] namely cyclopeptin (**3**), dehydrocyclopeptin (**4**), and cyclopenin (**5**; Figure 1 A). These benzodiazepinediones were predicted to be biosynthesized by the condensation of anthranilic acid and an amino acid,[7] a reaction which can be performed by a bimodular nonribosomal peptide synthetase (NRPS).[8] Thus, an NRPS which can combine anthranilic acid with either L-phenylalanine, L-tyrosine, or a derivative thereof would be able to form the 6,7-bicyclic precursor for the 3-phenyl-substituted 6,6-bicyclic core of the viridicatin scaffold. While there is a report of a putative NRPS proposed to form indolebenzodiazepinediones from anthranilic acid and L-tryptophan,[9] no NRPS which condenses anthranilic acid with other types of aromatic amino acids has been reported thus far. More importantly, details of the formation of the viridicatin biosynthetic intermediates, as well as the transformation of 6,7-benzodiazepinedione into 6,6-quinolone, still remain ill-defined to date. Thus, we set out to identify the biosynthetic enzymes responsible for the formation of viridicatin scaffold so that we can understand, in depth, the reaction mechanisms involved in this interesting biosynthetic process.

A previous study has shown that *A. nidulans* HKI 0410, a cognate strain of *A. nidulans* A1149, could produce the aspoquinolones A–D (**6**–**9**; Figure 1 B), which were proposed to be biosynthesized using **1** as an intermediate.[5a] Similarly, another strain of *A. nidulans*, that is, an endophytic strain of *A. nidulans* MA-143, was shown to produce a family of compounds having a viridicatin-type framework.[5e] Thus, we decided to examine the genome of *A. nidulans* A1149, a uracil auxotrophic, *nkuA* deletion strain of the genome-sequenced

[*] N. Ishikawa, Dr. H. Tanaka, Dr. F. Koyama, Prof. Dr. H. Noguchi, Prof. Dr. K. Watanabe
Department of Pharmaceutical Sciences
University of Shizuoka, Shizuoka 422-8526 (Japan)
E-mail: kenji55@u-shizuoka-ken.ac.jp
Prof. Dr. C. C. C. Wang
Departments of Pharmacology and Pharmaceutical Sciences and Chemistry, University of Southern California
Los Angeles, California 90089 (USA)
Prof. Dr. K. Hotta
School of Biosciences, The University of Nottingham
Malaysia Campus, Selangor 43500 (Malaysia)
Dr. H. Tanaka
Current address: Science Research Center, Oceanography Section
Kochi University, Kochi 780-8073 (Japan)

[**] We deeply thank Prof. Yoshitaka Hamashima for enlightening discussions on the radical reaction mechanism. We would like to express our appreciation for financial support from the Japan Society for the Promotion of Science (JSPS) through the "Funding Program for Next Generation World-Leading Researchers", initiated by the Council for Science and Technology Policy (No. LS103) (K.W.), and from the Industrial Technology Research Grant Program in 2009 (No. 09C46001a) from the New Energy and Industrial Technology Development Organization (NEDO) of Japan (K.W.). This work was also supported in part by Amano Enzyme Foundation (K.W.), Mochida Memorial Foundation for Medical and Pharmaceutical Research (K.W.), The Naito Foundation Japan (K.W.), Nagase Science and Technology Foundation Japan (K.W.), Takeda Science Foundation (K.W.), National Institute of General Medical Sciences grant PO1-GM084077 (C.C.C.W.), and the National Science Foundation, Emerging Frontiers in Research and Innovation MIKS grant 1136903 (C.C.C.W.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201407920.

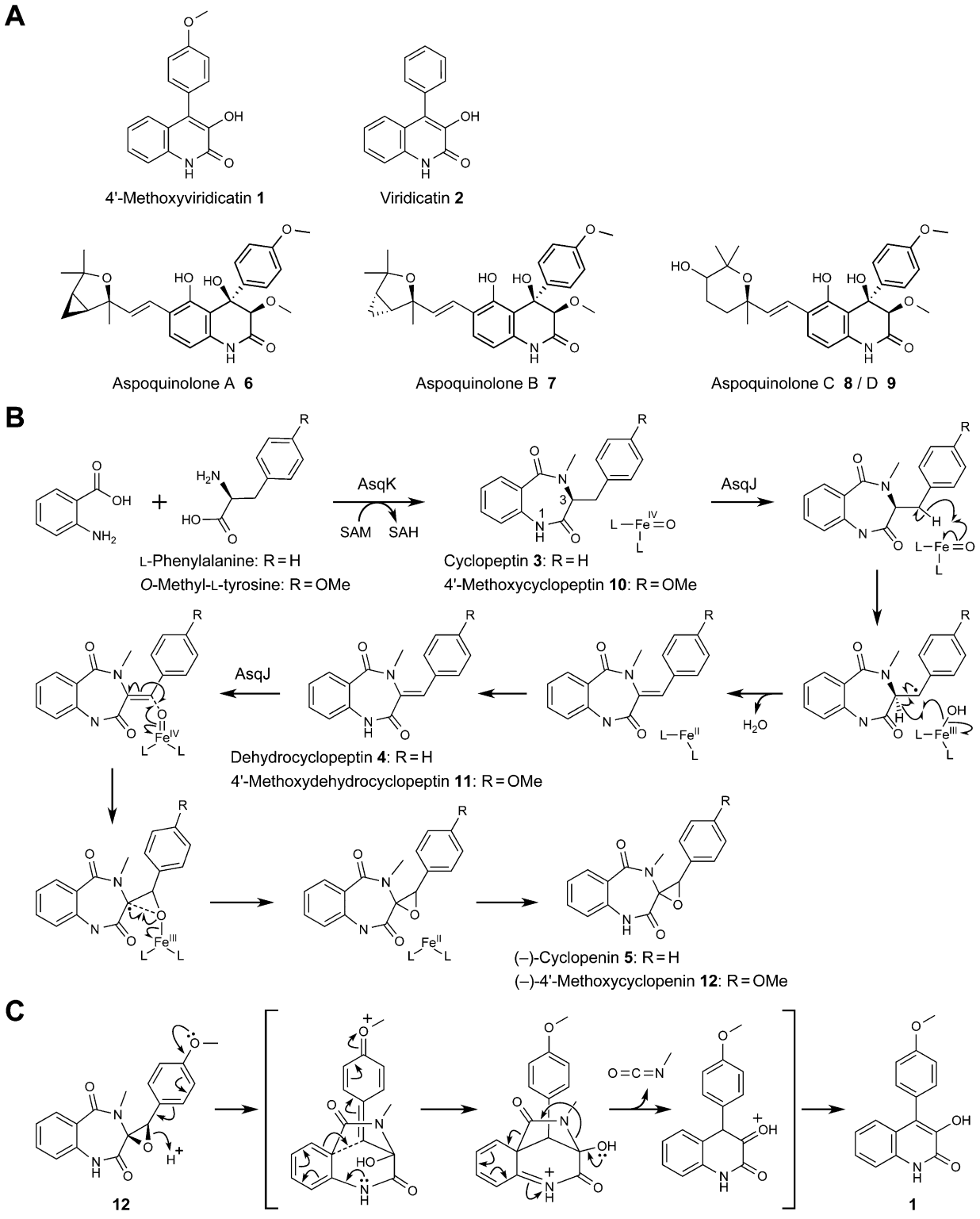

Figure 1. Proposed biosynthesis of 4′-methoxyviridicatin (**1**). A) Chemical structures of **1**, viridicatin (**2**), and aspoquinolones A–D (**6**–**9**). B) Proposed biosynthetic pathway of **1** involving two consecutive oxidation steps which are catalyzed by a single α-ketoglutarate-dependent dioxygenase, AsqJ. Note that the second step is an epoxidation reaction, the type of oxidation which is typically not performed by dioxygenase. The compounds **1** and **12** are newly isolated and characterized in this study. L = ligand, which comprises two histidines and an aspartic acid residue[11] (see Figure S40). C) Proposed mechanism of formation of the quinolone framework of **1** from **12**.

A. nidulans FGSC A4,[10] to locate the genes involved in the biosynthesis of **1**. Bioinformatics analysis of the *A. nidulans* genome and the chemical structure of **1**–**12** led us to consider a gene cluster containing the NRPS gene *asqK* and the prenyltransferase gene *asqH*, along with other genes encoding terpene cyclases and redox enzymes (see Figure S1 and Table

S1 in the Supporting Information), as a potential gene cluster for the biosynthesis of **1** and related quinolones. Transcriptional analysis of each gene in the cluster revealed that genes *asqE*, *F*, and *K* were expressed under conventional culturing conditions (see Figure S35). However, other genes appeared to remain silent, thus indicating that this gene cluster is another example of silent secondary metabolite biosynthetic gene clusters often found in fungal genomes. Since the majority of the genes are silenced,[12] and we could not detect the presence of **1** or related compounds in the culture of *A. nidulans* A1149, we decided to overexpress *asqK* in *A. nidulans* A1149 to ascertain what products this NRPS can produce. Upon introduction of an extra copy of *asqK*, placed on a plasmid into *A. nidulans* A1149, we were able to isolate cyclopeptins from the culture extracts of this modified *A. nidulans* A1149 strain. This strain was able to produce **3** and 4′-methoxycyclopeptin (**10**) when L-phenylalanine and *O*-methyl-L-tyrosine, respectively, were introduced (see Figure S2). To evaluate the extender amino acid specificity of the NRPS AsqK, we performed HPLC analysis of the two culture extracts and discovered that *O*-methyl-L-tyrosine was nearly tenfold more efficiently incorporated into the benzodiazepinedione framework than L-phenylalanine, with a yield of 20 mg L^{-1} for the formation of **10** as compared to a yield of 2 mg L^{-1} for **3**. This result indicated that *O*-methyl-L-tyrosine is the actual substrate for AsqK, and suggested strongly that the *asq* gene cluster is likely responsible for the biosynthesis of **1**, the key motif of **6**–**9**. In addition, the observation of the formation of **10** by AsqK clarifies a previous question[8] regarding the origin of the *S*-adenosyl-L-methionine-derived *N*-methyl group in the cyclopeptin skeleton. With putative standalone methyltransferase genes in the cluster not transcribed at an appreciable level (see Figure S35), the *N*-methyl group appears to be introduced by the embedded methyltransferase domain of AsqK.

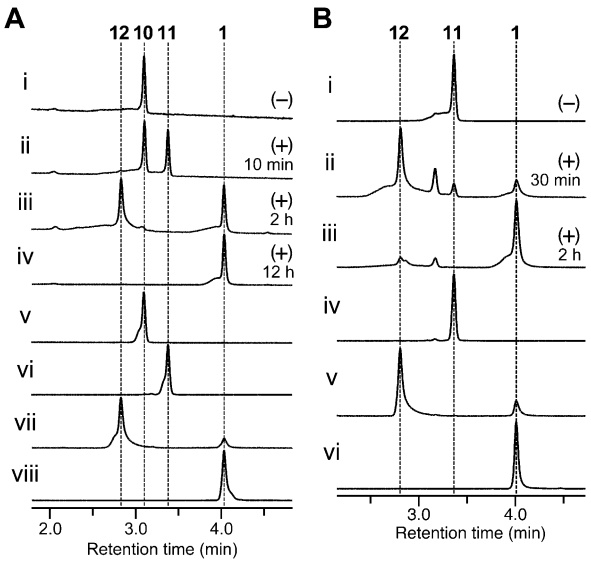

Figure 2. In vitro analysis of the activity of recombinant AsqJ against different 4′-methoxylated substrates. Detailed reaction conditions are described in the Supporting Information. All traces were monitored at $\lambda = 280$ nm. A) HPLC profiles of: i) the reaction mixture containing heat-inactivated AsqJ, indicated by (−), with **10** as a negative control; ii) the reaction mixture containing active AsqJ, indicated by (+), and **10**, showing the formation of **11** after 10 min of incubation; iii) the reaction mixture containing active AsqJ and **10**, showing the formation of **12** and then **1** after two hours of incubation; iv) the reaction mixture containing active AsqJ and **10**, showing complete conversion of **10** into **1** after 12 h of incubation. The authentic references are given for **10** (v), **11** (vi), **12** (vii), and **1** (viii). B) HPLC profiles of: i) the reaction mixture containing heat-inactivated AsqJ with **11**; ii) the reaction mixture containing AsqJ and **11**, showing the formation of **12** after 30 min of incubation; iii) the reaction mixture containing AsqJ and **11**, showing the formation of **1** after two hours of incubation. The authentic references are shown for **11** (iv), **12** (v), and **1** (vi).

Next, to examine the involvement of each of the predicted redox enzymes, AsqE, F, G, I, J, and L, in the quinolone formation, we attempted in vivo bioconversion experiments using **10** (see Figures S17 and S18 and Tables S6 and S7) as a fed substrate. For this study, we generated a series of *A. nidulans* A1149 strains, expressing each of the six genes, from an expression plasmid (see the Supporting Information for details). These in vivo assays revealed that the new compound **11** (Figure 1 A), was formed in the presence of AsqJ (see Figure S4). High-resolution electrospray ionization (HRESI) LC-MS and ^{1}H NMR, ^{13}C NMR spectrometric analyses identified **11** as 4′-methoxydehydrocyclopeptin (see Figures S19–S24 and Table S8). Thus, AsqJ is capable of performing dehydrogenation on **10** to form a double bond between C_α and C_β of the *O*-methyltyrosine side chain to form **11**. To confirm the function of AsqJ and understand the detailed reaction mechanism for the formation of **11**, we characterized the activity of AsqJ against **10** in vitro (Figure 2 A, i versus ii). Recombinant AsqJ was produced successfully in *Escherichia coli* and purified to homogeneity (see Figure S38 and the Supporting Information). We performed further in vitro analysis on the purified AsqJ, where the dependence of the activity of AsqJ on cofactors required by an α-ketoglutarate-dependent dioxygenase[13] was examined. The result indicated that lack of α-ketoglutarate or inclusion of ethylenediaminetetraacetic acid in the reaction mixture substantially diminished the ability of AsqJ to convert **10** into **11**, thus confirming that the dioxygenase activity of AsqJ was responsible for the formation of the double bond in **11** (see Figure S39).

To our surprise, the in vitro analysis of AsqJ revealed that a longer incubation of the reaction mixture (two hours as compared to ten minutes) resulted in the total loss of **11** and the formation of an another compound (**12**; see Figure 1 A) which appeared as a clear UV peak, and whose structure was identified to be that of (−)-4′-methoxycyclopenin, an epoxidized product of **11** (Figure 2 A, iii; see Figures S25–S29 and Table S9). Furthermore, a clear peak representing **1** (see Figures S30–S34 and Table S10) was also observed. After a prolonged incubation (12 h), all of the products were converted into **1** (Figure 2 A, iv). The sequential nature of the transformation was corroborated by a separate in vitro reaction in which AsqJ converted **11** into **12** then into **1** (Figure 2 B). This unexpected successive conversion of **10** into **12** via **11** revealed that AsqJ is a very unique dioxygenase which is capable of catalyzing radical-mediated dehydrogenation and epoxidation reactions sequentially on a 6,7-benzodiazepinedione substrate in the 4′-methoxyviridicatin biosyn-

thetic pathway. Another unexpected observation from the assay, namely the formation of **1**, was confirmed to proceed non-enzymatically by observing the spontaneous and quick conversion of **12** into **1** in an aqueous buffer at 30 °C (see Figure S41). Similar to the previously proposed mechanism for the formation of **2**,[8] we propose the reaction mechanism of the formation of **1** to involve opening of the epoxide in **12**, a step is initiated by donation of the methoxy oxygen lone pair of electrons. This opening results in the conversion of the 6,7-bicyclic system into the 6,6-bicyclic quinolone of **1** upon elimination of carbon dioxide and methylamine in the form of methyl isocyanate (Figure 1 C).

Considering that AsqK is capable of producing **3**, we examined wheter the Asq enzymes are capable of biosynthesizing the non-4′-methoxylated series of products. Analyses of the *asq* genes similar to those performed earlier (Figure 3) revealed that AsqJ is indeed capable of converting **3** into **4** (see Figure S7–S9 and Table S4) and **5** (see Figure S10–S14 and Table S5) in a sequential fashion. However, we were not able to observe the AsqJ-catalyzed conversion of **5** into **2** (see Figures S15 and S16). While it is known that **5** can be converted into **2** non-enzymatically, the rate of conversion is far slower than that of the conversion of **12** into **1**.[4,6b,14] This slow conversion is probably the reason a viridicatin-producing fungus carries an enzyme called a cyclopenase.[6a,14,15] Unfortunately, cyclopenase has only been characterized in a fractionated cell extract and has not been isolated in the pure form. Thus, detailed biochemical or mechanistic information is not known about this enzyme at this time. Cyclopenase is absent from the *asq* cluster, presumably because the conversion of **12** into **1** proceeds spontaneously.

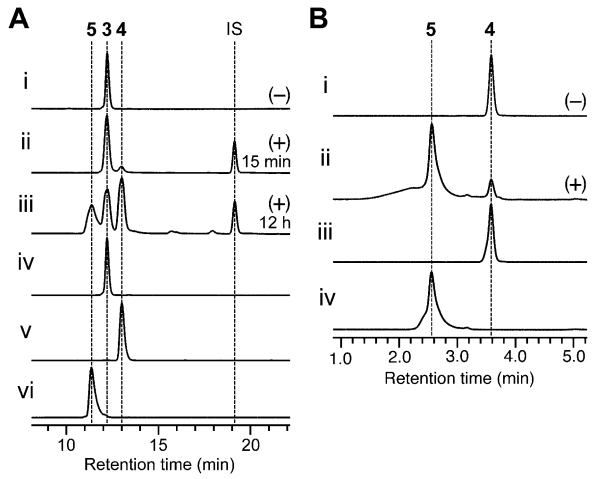

Figure 3. In vitro analysis of the activity of AsqJ against different non-4′-methoxylated substrates. Detailed reaction conditions are described in the Supporting Information. Peaks from separate runs were standardized by scaling together the height of the IS peak (*N*-Boc-L-tryptophan methyl ester). All traces were monitored at $\lambda = 280$ nm. A) HPLC profiles of: i) the reaction mixture containing heat-inactivated AsqJ, indicated by (−), with **3** as a negative control; ii) the reaction mixture containing active AsqJ, indicated by (+), and **3**, showing the formation of **4** after 15 min of incubation; iii) the reaction mixture containing active AsqJ and **3**, showing the formation of **4** and **5** after 12 h of incubation. The authentic references are shown for **3** (iv), **4** (v), and **5** (vi). B) HPLC profiles of: i) the reaction mixture containing heat-inactivated AsqJ with **4**; ii) the reaction mixture containing AsqJ and **4**, showing the formation of **5**. The authentic references are shown for **4** (iii) and **5** (iv). IS = internal standard (*N*-Boc-L-tryptophan methyl ester).

In conclusion, we identified the gene cluster in *A. nidulans* that is responsible for the biosynthesis of 4′-methoxylated 6,7-benzodiazepinediones and 4′-methoxyviridicatin, which likely act as a precursor of quinolone natural products, such as aspoquinolones,[5a] peniprequinolones,[5b] penigequinolones,[5c] and yaequinolones.[5d] As such, our study provides new insight into how quinolone and quinolinone alkaloids are biosynthesized in fungi. Further characterization of the remaining genes in the cluster is currently ongoing to determine the exact identity of quinolone products this cluster is responsible for biosynthesizing. Most interestingly, however, our study revealed that the dioxygenase AsqJ to be responsible for the two-step oxidation of the NRPS product, where the latter epoxidation of the compound sets up the system for the conversion of a 6,7-bicyclic skeleton into a 6,6-bicyclic quinolone framework. During the first half of the transformation, AsqJ creates a double bond in the 6,7-bicyclic substrate by a radical-mediated dehydrogenation. Then, during the latter half of the process, AsqJ converts the double bond it just formed into an epoxide. AsqJ is the first reported fungal iron- and α-ketoglutarate-dependent, non-heme dioxygenase involved in alkaloid biosynthesis which not only performs two sequential oxidation reactions on a single substrate but also catalyzes an epoxidation reaction. While there are known examples of dioxygenases performing sequential reactions on a substrate, such as deacetoxycephalosporin/deacetylcephalosporin C synthase from cephalosporin biosynthesis[16] and flavonol synthase in flavonoid biosynthesis,[17] the only other example of a dioxygenase catalyzing sequential dehydrogenation–epoxidation reactions was found in the pentalenolactone sesquiterpenoid biosynthesis in *Streptomyces*.[18] Moreover, unlike the pentalenolactone biosynthesis, epoxidation of **11** is not the end point of the biosynthesis but the initiation of an efficient elimination-rearrangement of the 6,7-bicyclic benzodiazepinedione core to form the 6,6,-bicyclic viridicatin framework. While dioxygenases are known to catalyze a wide variety of redox reactions,[19] AsqJ is rare in that it catalyzes an epoxidation reaction. Typically, monooxygenases, such as cytochrome P450s and flavin-containing monooxygenases, are responsible for the formation of epoxides in different metabolites.[20] Apart from the pentalenolactone biosynthetic enzymes described above, the only reported case of a dioxygenase catalyzing an epoxidation reaction involves the biosynthesis of a bacterial nonribosomal peptide antibiotic dapdiamide.[21] Our finding indicates that this dioxygenase-catalyzed epoxidation is the key step in the rearrangement of benzodiazepinediones into viridicatins in alkaloid biosynthesis, thus hinting at the potential of this type of dioxygenase as a unique catalyst for synthesizing various 6,6-quinolones from benzodiazepinediones prepared from anthranilic acid with various different amino acids.

Received: August 4, 2014
Published online: September 22, 2014

Keywords: alkaloids · enzymes · gene expression · natural products · oxidations

[1] A. Ahmed, M. Daneshtalab, *J. Pharm. Pharm. Sci.* **2012**, *15*, 52 – 72.
[2] T. P. Cushnie, B. Cushnie, A. J. Lamb, *Int. J. Antimicrob. Agents* **2014**, DOI:10.1016/j.ijantimicag.2014.06.001.
[3] a) K. G. Cunningham, G. G. Freeman, *Biochem. J.* **1953**, *53*, 328 – 332; b) A. Ciegler, C. T. Hou, *Arch. Mikrobiol.* **1970**, *73*, 261 – 267.
[4] H. W. Smith, H. Rapoport, *J. Am. Chem. Soc.* **1969**, *91*, 6083 – 6089.
[5] a) K. Scherlach, C. Hertweck, *Org. Biomol. Chem.* **2006**, *4*, 3517 – 3520; b) M. Kusano, H. Koshino, J. Uzawa, S. Fujioka, T. Kawano, Y. Kimura, *Biosci. Biotechnol. Biochem.* **2000**, *64*, 2559 – 2568; c) Y. Kimura, M. Kusano, H. Koshino, J. Uzawa, S. Fujioka, K. Tani, *Tetrahedron Lett.* **1996**, *37*, 4961 – 4964; d) R. Uchida, R. Imasato, H. Tomoda, S. Omura, *J. Antibiot.* **2006**, *59*, 652 – 658; e) C. Y. An, X. M. Li, H. Luo, C. S. Li, M. H. Wang, G. M. Xu, B. G. Wang, *J. Nat. Prod.* **2013**, *76*, 1896 – 1901.
[6] a) M. Luckner, *Eur. J. Biochem.* **1967**, *2*, 74 – 78; b) J. D. White, M. J. Dimsdale, *J. Chem. Soc. D* **1969**, 1285b – 1286.
[7] L. Nover, M. Luckner, *Eur. J. Biochem.* **1969**, *10*, 268 – 273.
[8] C. T. Walsh, S. W. Haynes, B. D. Ames, X. Gao, Y. Tang, *ACS Chem. Biol.* **2013**, *8*, 1366 – 1382.
[9] W. B. Yin, A. Grundmann, J. Cheng, S. M. Li, *J. Biol. Chem.* **2009**, *284*, 100 – 109.
[10] T. Nayak, E. Szewczyk, C. E. Oakley, A. Osmani, L. Ukil, S. L. Murray, M. J. Hynes, S. A. Osmani, B. R. Oakley, *Genetics* **2006**, *172*, 1557 – 1566.
[11] E. L. Hegg, L. Que, Jr., *Eur. J. Biochem.* **1997**, *250*, 625 – 629.
[12] A. A. Brakhage, V. Schroeckh, *Fungal Genet. Biol.* **2011**, *48*, 15 – 22.
[13] R. P. Hausinger, *Crit. Rev. Biochem. Mol. Biol.* **2004**, *39*, 21 – 68.
[14] M. Luckner, K. Winter, J. Reisch, *Eur. J. Biochem.* **1969**, *7*, 380 – 384.
[15] a) M. Luckner, *J. Nat. Prod.* **1980**, *43*, 21 – 40; b) S. Wilson, I. Schmidt, W. Roos, W. Fürst, M. Luckner, *Z. Allg. Mikrobiol.* **1974**, *14*, 515 – 523; c) S. Wilson, M. Luckner, *Z. Allg. Mikrobiol.* **1975**, *15*, 45 – 51.
[16] M. D. Lloyd, S. J. Lipscomb, K. S. Hewitson, C. M. Hensgens, J. E. Baldwin, C. J. Schofield, *J. Biol. Chem.* **2004**, *279*, 15420 – 15426.
[17] R. Lukačin, F. Wellmann, L. Britsch, S. Martens, U. Matern, *Phytochemistry* **2003**, *62*, 287 – 292.
[18] M. J. Seo, D. Zhu, S. Endo, H. Ikeda, D. E. Cane, *Biochemistry* **2011**, *50*, 1739 – 1754.
[19] a) F. H. Vaillancourt, E. Yeh, D. A. Vosburg, S. E. O'Connor, C. T. Walsh, *Nature* **2005**, *436*, 1191 – 1194; b) R. M. Cicchillo, H. Zhang, J. A. Blodgett, J. T. Whitteck, G. Li, S. K. Nair, W. A. van der Donk, W. W. Metcalf, *Nature* **2009**, *459*, 871 – 874; c) Y. Matsuda, T. Awakawa, T. Wakimoto, I. Abe, *J. Am. Chem. Soc.* **2013**, *135*, 10962 – 10965; d) S. D. Wong, M. Srnec, M. L. Matthews, L. V. Liu, Y. Kwak, K. Park, C. B. Bell 3rd, E. E. Alp, J. Zhao, Y. Yoda, S. Kitao, M. Seto, C. Krebs, J. M. Bollinger, Jr., E. I. Solomon, *Nature* **2013**, *499*, 320 – 323.
[20] a) E. M. Isin, F. P. Guengerich, *Biochim. Biophys. Acta Gen. Subj.* **2007**, *1770*, 314 – 329; b) R. D. Ceccoli, D. A. Bianchi, D. V. Rial, *Front. Microbiol.* **2014**, *5*, 25; c) Y. Tsunematsu, N. Ishikawa, D. Wakana, Y. Goda, H. Noguchi, H. Moriya, K. Hotta, K. Watanabe, *Nat. Chem. Biol.* **2013**, *9*, 818 – 825; d) Y. Hu, D. Dietrich, W. Xu, A. Patel, J. A. Thuss, J. Wang, W. B. Yin, K. Qiao, K. N. Houk, J. C. Vederas, Y. Tang, *Nat. Chem. Biol.* **2014**, *10*, 552 – 554.
[21] M. A. Hollenhorst, S. B. Bumpus, M. L. Matthews, J. M. Bollinger, Jr., N. L. Kelleher, C. T. Walsh, *J. Am. Chem. Soc.* **2010**, *132*, 15773 – 15781.

