Organic LETTERS

Letter

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Characterization of QmnD3/QmnD4 for Double Bond Formation in Quartromicin Biosynthesis

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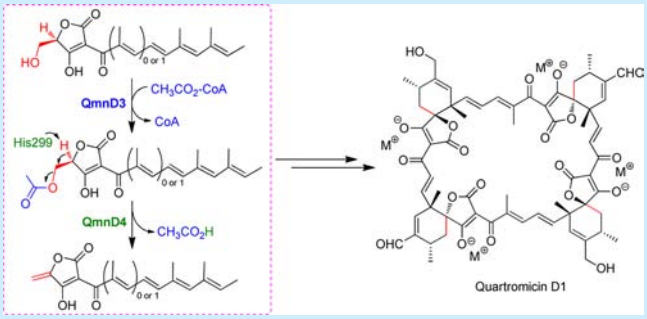
Supporting Information

ABSTRACT: In this work, two enzymes responsible for the biogenesis of possible [4 + 2] reaction precursors in the quartromicin biosynthetic pathway were characterized: acetylation of **1** to yield **2** was catalyzed by QmnD3, and subsequent acetic acid elimination of **2** to form double bond product **3** was catalyzed by QmnD4. Site-directed mutagenesis assay of QmnD3 and QmnD4 was investigated, and a general base-catalyzed mechanism for QmnD4 is proposed.



Spirotetronates are diverse polyketide natural products that show a wide range of biological effect. The antitumor compounds tetrocarcin A and kijanimicin,[1,2] the antibacterial compounds chlorothricin and abyssomicin,[3,4] the cholecystokinin B inhibitor tetronothiodin,[5] and the GRP78/Bip molecular chaperone down-regulator versipelostatin[6] are well-known examples. These compounds all possess a polycyclic aglycon that features a characteristic spirotetronate moiety (tetronic acid spiro-linked to a cyclohexene ring). Quartromicins (QMNs, Figure 1) represent an unusual group of spirotetronates produced by *Amycolatopsis orientalis* with antiviral activity against several viruses including HIV.[7] The four spirotetronate units harbored in QMNs and the C2-symmetric aglycon structure distinguish them from other spirotetronates (Supplementary Figure S1). Although more than one decade has passed since their discovery, QMNs are still challenging synthetic targets remaining to be conquered despite many efforts.[8]

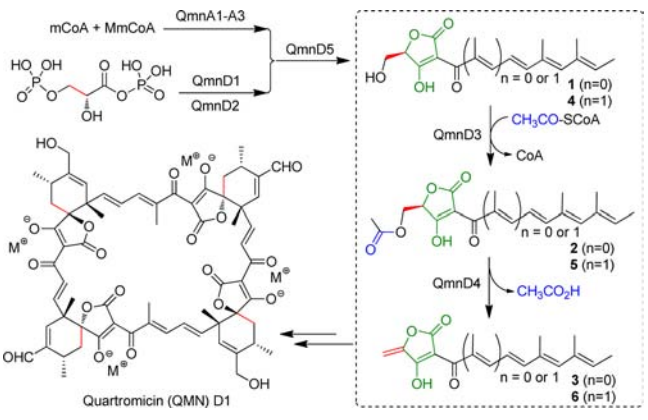


Figure 1. Proposed biosynthetic pathway of QMNs. The process for the formation of the exocyclic double bond is boxed, and the tetronate moiety is highlighted in green.

From years of biosynthetic studies,[9−11] a common picture for the pathway of spirotetronate natural products now can be drawn as follows: (i) assembly of the core polyketide chain by type I polyketide synthase (PKS), (ii) construction of the tetronate intermediate (4-hydroxy-[5*H*]furan-2-one, Figure 1) by incorporation of the glycerol-derived three-carbon unit, (iii) dehydration of the tetronate intermediate and ensuing [4 + 2] cyclization reaction to form the aglycone core structure, and (iv) further tailing reactions such as hydroxylation and glycosylation to yield the final product. Among these steps, the dehydration process to form the [4 + 2] precursor and the possible [4 + 2] cyclization step are the most challenging questions and rarely understood. We were interested in the biosynthetic logic of spirotetronate antibiotics for several years,[9,11] especially in elucidating how the spirotetronate moieties were formed. In our earlier study on the biosynthesis of QMN,[11] we have reconstituted the formation of tetronate intermediate **1** *in vitro* and identified that one set of polyketide synthases are responsible for the production of two alternative polyketide chains, **1** and **4**, which were both proposed to be incorporated into the final product (Figure 1). In this work, we show here that the possible [4 + 2] cycloaddtion precursor **3** is derived from **1** by acetyltransferase QmnD3 and α/β hydrolase fold protein QmnD4 in an acetyl-CoA dependent manner (Figure 1, boxed part): first, the O-acetylation of the hydroxyl group of tetronate by QmnD3 and second, acetic acid elimination yielding the exocyclic double bond by QmnD4. This two-step enzymatic logic is similar to the recently reported

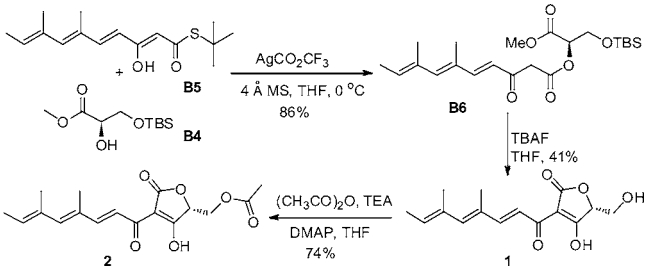
Received: January 13, 2014
Published: February 28, 2014

dx.doi.org/10.1021/ol500111n | *Org. Lett.* 2014, 16, 1578−1581

results in agglomerin biosynthesis by Leadlay and Sun.[12] Further biochemical assay showed that the conserved HxxxD motif in QmnD3 is essential for the acyltransferase activity. The conserved Ser189, which is typically the catalytic residue in α/β hydrolase family enzymes, played no role in QmnD4, which suggests a different active site topology and catalytic mechanism to canonical hydrolase enzymes.

With the intention to explore the biosynthetic steps after the tetronate ring has formed,[11] **1** was obtained through chemical synthesis (Scheme 1, methods in Supporting Information). The

Scheme 1. Synthetic Scheme for Compounds 1 and 2[a]



[a]Reaction details are provided in Supporting Information.

three-carbon unit was endogenously determined to be (*R*)-1,3-bisphospoglycerate, so **B4** was synthesized from the commercially available (*R*)-enantiomer of methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate. β-Ketothioester **B5** was synthesized as described by Ley.[13] **B6** was obtained from **B4** and **B5** by an $AgCO_2CF_3$-catalyzed transesterification.[14] Cyclization and deprotection reactions were carried out in a "one-pot" manner under TBAF to afford the expected product **1**.[15]

It was proposed that the QMN core structure was formed from 2 equiv of **3** and **6** through four intermolecular [4 + 2] cyclizations (Figure 1).[11,16] Thus, how the exocyclic double bond was formed became the first question. Interestingly, comparative gene cluster analysis of tetronate related natural products showed that there was a strict correlation between the presence of *qmnD3/qmnD4* homologues and the dehydration step and inspired the proposal that QmnD3 and QmnD4 catalyze the dehydration reaction of **1** to the exocyclic double bond intermediate **3** (Supplementary Figure S2).

QmnD3 is an acyltransferase-like protein and homologous to chloramphenicol acetyltransferase (CAT) and the C-terminal catalytic domain of E2p (dihydrolipoyltransacetylase) of the pyruvate dehydrogenase multienzyme complex.[17,18] Both enzymes are involved in acetyl group transfer. Particularly, the former enzyme catalyzes the transfer of an acetyl group from acetyl-CoA to the primary hydroxyl of chloramphenicol for bacterial resistance. Importantly, a conserved $H_{227}xxxD_{231}$ motif was found when QmnD3 and homologues were aligned to CATs, which is the catalytic active site in type III variant of CAT from *E. coli* (Supplementary Figure S3).[17] The information gathered strongly suggested QmnD3 might proceed in an acetyl-CoA dependent manner. Notably, malonylation of hydroxyl group during the generation of double bond in phoslactomycin and fostriecin biosynthesis further strengthen this hypothesis.[19] QmnD4 belongs to α/β hydrolase family, but its poor sequence similarity to any protein of known function hindered further analysis. QmnD3 and QmnD4 were heterologously overexpressed in *E. coli* BL21 (DE3) and purified to homogeneity, respectively (Supplementary Figure S4). We incubated substrate **1** and acetyl-CoA with QmnD3 first. A new product was observed by HPLC analysis (Figure 2A). LC−MS analysis gave a signal of 321.2 (m/z [M + H]$^+$), which suggested it was the acetylation product **2**. When acetyl-CoA or QmnD3 was omitted from the reaction or denatured QmnD3 was used for the assay, no product could be detected. When QmnD4 was added into the above system, another new product appeared (Figure 2B). The LC−MS signal of 261.2 (m/z [M + H]$^+$) is consistent with the double bond product **3**. Further investigation of the reaction time course indicated that QmnD3 acetylates the hydroxyl group of **1** to produce the ester **2**, and **2** was converted to **3** by QmnD4 (Figure 2C). This conclusion is also supported by the similar UV spectra of **1**, **2**, and **3** (Supplementary Figure S5). To further validate the above results, **2** was synthesized from **1** as reference, but the synthesis of **3** was unsuccessful because of the instability of this compound during concentration, although the transformation of **2** to **3** under DBU was facile (data not shown). The same retention time and LC/MS result proved that the first product was compound **2** (Figure 2B). The transformation of **2** to **3** was monitored using ^{1}H NM further. After incubation of QmnD4 and substrate **2** for 1 h (Methods in Supporting Information), two strong NMR signals were observed at 5.23 ppm (d, 2.5 Hz) and 5.01 ppm (d, 2.5 Hz), which are characteristic of a terminal olefin. A signal at 1.92 ppm representing the newly formed methyl group of acetic acid was also observed (Supplementary Figure S6). The results here firmly established that the formation of exocyclic double bond intermediate **3** from **1** was catalyzed by QmnD3/QmnD4 via acetylation and acetic acid elimination reactions.

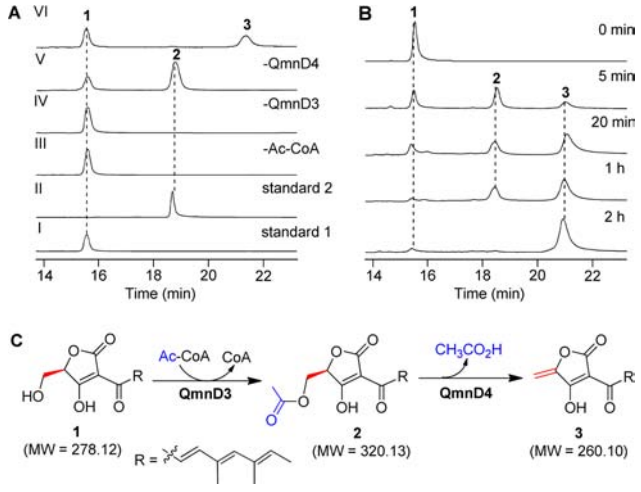


Figure 2. *In vitro* assay of QmnD3 and QmnD4. (A) HPLC analysis of **1** under QmnD3/QmnD4 and acetyl-CoA: (I) substrate **1**; (II) authentic **2**; reaction without acetyl-CoA (III), QmnD3 (IV), and QmnD4 (V); (VI) full reaction. (B) Time course of enzymatic reaction. (C) Double bond formation catalyzed by QmnD3 and QmnD4.

After the initial experiment, the reactions catalyzed by QmnD3 and QmnD4 were explored in more detail. Given the fact that the HxxxD motif plays an essential role for activity of CATs,[17] His-227 and Asp-231 of QmnD3 were mutated to Ala, respectively. Additionally, Arg-103, Arg-143, Ser-178, His-181, Asp-185, and Asp-210, which were conserved only in QmnD3 homologues, were mutated into Ala to identify other potential key residues for catalysis. Biochemical assays of all of these mutants show that mutant H227A abolished and D231A

dx.doi.org/10.1021/ol500111n | *Org. Lett.* 2014, 16, 1578−1581

decreased the acetylation activity (Figure 3A), suggesting that the $H_{227}xxxD_{231}$ motif is essential for QmnD3 activity. This is

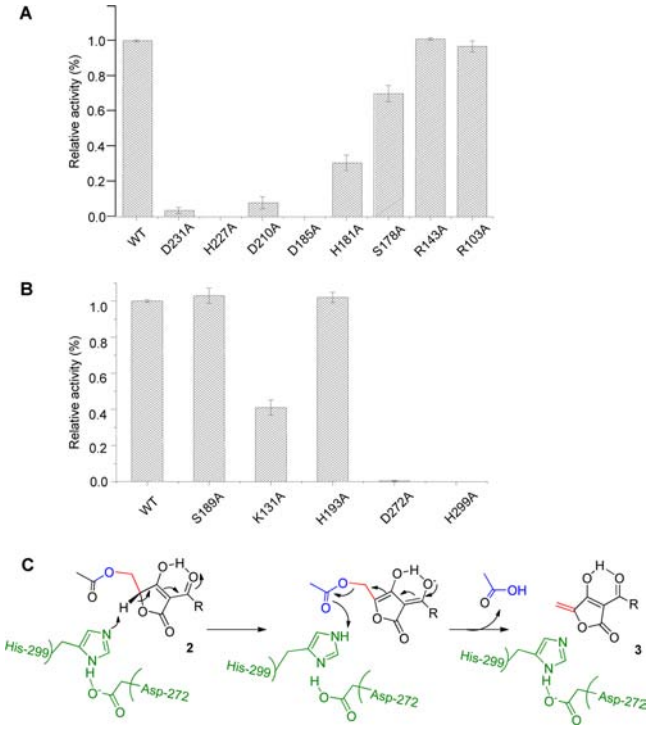


Figure 3. Mutagenesis assay of QmnD3 and QmnD4. (A) QmnD3 mutants activities are shown as percentage of the wild type. (B) QmnD4 mutants activities. (C) Proposed general base mechanism for QmnD4.

consistent with the previous proposal that His-199 of CAT from *E. coli* as the general base to abstract a proton from the hydroxyl group. Notably, Asp-185, which is conserved between QmnD3 homologues and CATs, is also essential for QmnD3 activity. The decreased activity of S178A, H181A, and D210A indicated that these residues are important but not essential for QmnD3 function. The cosubstrate specificity of QmnD3 was also investigated. Not surprisingly, acetyl-CoA is the best donor substrate (k_{cat}/K_m = 185 mM^{-1} min^{-1}), but also propionyl-CoA (k_{cat}/K_m = 78 mM^{-1} min^{-1}) and butyryl-CoA (though the efficiency is very low, k_{cat}/K_m = 0.8 mM^{-1} min^{-1}) could be used as acyl donors (Supplementary Figure S7).

Next, we explored the α/β hydrolase fold protein QmnD4. The α/β hydrolase fold superfamily is a large group of proteins that encompass broad catalytic activities.[20] This family is characterized by a twisted β-sheet flanked by α-helices on both sides and by a catalytic triad consisting of a His, a nucleophile, and an acidic residue. Although having low sequence homology, the catalytic triad of these enzymes adopt conserved spatial positions, and the catalytic nucleophile residue was always placed in a conserved sharp strand-loop-helix turn motif.[20a] Most α/β hydrolases use a catalytic triad to catalyze the hydrolysis of C−O, C−N, C−S, C−X (X for halogen), and C−C bonds through a nucleophilic attack mechanism.[21] However, whether QmnD4 uses a similar catalytic triad for catalysis as in canonical α/β hydrolase enzymes is uncertain. Thus, the catalytic triad (Ser-189, Asp-272, and His-299) were mutated to Ala based on a homology structure of QmnD4 modeled by PHYRE (Protein Homology/analogy Recognition Engine v2.0) protein folding program (Supplementary Figure S8).[22] Biochemical assay showed that H299A mutant completely abolished and D272A mutant largely decreased the activity (Figure 3B). The full activity of S189A mutant suggests that the nucleophile Ser-189, which is located in the nucleophile elbow position, is not involved in catalysis. These results suggest that QmnD4 adopted a different active site topology to typical α/β hydrolase enzymes and are consistent with the request of a base (His-299 in QmnD4) to initiate an elimination reaction than a nucleophile to conduct hydrolysis. Thus, a catalytic mechanism of QmnD4 is proposed: His-299 abstracts a proton from the substrate **2** to generate an enol intermediate that can be stabilized by delocalization, and leaving group departure yields the double bond product **3** (Figure 3C). Acetylation of hydroxyl group by QmnD3 is an essential step for elimination activity of QmnD4 (Figure 2B). This prior acetylation provides two chemical advantages for the elimination reaction catalyzed by QmnD4. First, the abstracted hydrogen atom of **2** is apparently more acid than its counterpart **1** and thus can be removed easier. Our computations using density functional theory indicate that the pK_a value of the hydrogen atom of **2** is 9.8, whereas that of the corresponding hydrogen atom of **1** is 11.1 (Method in Supporting Information). Second, acetylation results in a better leaving group (acetic acid versus water) and facilitates the elimination by this activation/elimination strategy whether it occurs through a stepwise or a concerted mechanism (although a stepwise mechanism is proposed here). Additionally, the minor or lack of effects on activity of mutants K131A and H193A (Supplementary Figure S9) highlight the importance of His-299 as the general base.

In summary, we have validated that QmnD3 and QmnD4 catalyze the formation of the double bond tetronate intermediates that are the precursors for the formation of QMN. These results not only set the stage to explore the [4 + 2] steps in QMN but also confirmed the strategy for the exocyclic double bond formation in tetronate related natural products. A general base-catalyzed mechanism of QmnD4 was suggested based on a site-directed mutagenesis assay. Comparatively, once the nucleophilic residue Ser of another α/β hydrolase fold enzyme, *Candida Antarctica* lipase B (CALB, EC 3.1.1.3), is mutated into Ala or Gly, the nucleophilic functionality will be removed and enable the His residue acting as a general base to initiate C−C bond construction.[23] The ability of QmnD4 to abstract a proton from the α-position of the carbonyl compound gives it potential as new starting material for further protein engineering.[24]

■ ASSOCIATED CONTENT

Supporting Information

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■ AUTHOR INFORMATION

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully thank Prof. Yikang Wu, Ang Li, and Ran Hong of SIOC for helpful suggestions and discussions on the chemical synthesis of substrates and Dr. Wen-Xian Lan for the NMR analysis and acknowledge the financial support of the 973 Program (2010CB833200 and 2012CB721100) and the NSFC (21072214 and 31330003).

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