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Menaquinone Biosynthesis: Formation of Aminofutalosine Requires a Unique Radical SAM Enzyme

Nilkamal Mahanta,[†] Dmytro Fedoseyenko,[†] Tohru Dairi,[‡] and Tadhg P. Begley*[,†]

[†]Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States
[‡]Graduate School of Engineering, Hokkaido University, Sapporo 060-8628, Japan

Supporting Information

ABSTRACT: Menaquinone (MK, vitamin K_2) is a lipid-soluble molecule that participates in the bacterial electron transport chain. In mammalian cells, MK functions as an essential vitamin for the activation of various proteins involved in blood clotting and bone metabolism. Recently, a new pathway for the biosynthesis of this cofactor was discovered in *Streptomyces coelicolor* A3(2) in which chorismate is converted to aminofutalosine in a reaction catalyzed by MqnA and an unidentified enzyme. Here, we reconstitute the biosynthesis of aminofutalosine and demonstrate that the missing enzyme (aminofutalosine synthase, MqnE) is a radical SAM enzyme that catalyzes the addition of the adenosyl radical to the double bond of 3-[(1-carboxyvinyl)oxy]benzoic acid. This is a new reaction type in the radical SAM superfamily.

Menaquinone (**8**, vitamin K) is a lipid-soluble quinone that shuttles electrons between membrane-bound redox enzymes in the respiratory chain in bacteria.[1] It is also an essential vitamin in humans, where it functions as a cosubstrate in the carboxylation of glutamic acid residues in proteins involved in blood clotting[2] and bone formation.[3] Until recently, it was believed that the bacterial biosynthesis of menaquinone from chorismate was well understood.[4,5] The observation that some strains of *Streptomyces* (e.g., *S. coelicolor* A3(2) and *S. avermitilis*) do not possess orthologs of the known menaquinone biosynthetic genes, however, suggested the existence of an alternative pathway. Using a combination of bioinformatics, labeling studies, gene deletions, and *in vitro* biochemical assays, the general features of this new pathway in *S. coelicolor* A3(2) were recently determined[6−8] (Figure 1). In this biosynthetic pathway, MqnA (SCO4506) plays an undefined role in catalyzing the coupling of chorismate (**1**), adenosine (**2**), and pyruvate (**3**) to form aminofutalosine (**4**) using unknown chemistry.[6] Aminofutalosine (**4**) is then converted to compound **5** by aminofutalosine hydrolase (MqnB) or by a combination of aminofutalosine deaminase and MqnB.[9,10] Cyclization of **5** by MqnC gives compound **6**,[11] which is then converted to 1,4-dihydroxy-6-naphthoic acid (**7**) by MqnD. Bioinformatics analysis suggests that **7** is converted to menaquinone **8** in *S. coelicolor* A3(2) in reactions catalyzed by SCO4491 (prenylation), SCO4556 (methylation), and SCO4490 or SCO4492 (decarboxylation).

Three possible pathways for the formation of **4** were considered (Figure 2). Paths A and B require a thiamin pyrophosphate dependent ketoacid decarboxylase, while path C requires a radical SAM enzyme. To determine which path was used, we analyzed the gene neighborhood of the identified menaquinone biosynthetic genes for additional genes encoding

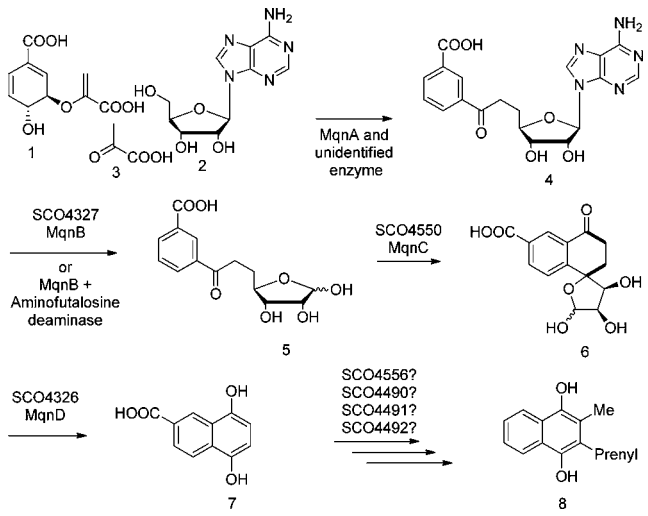

Figure 1. Futalosine-dependent pathway to menaquinone, **8**.

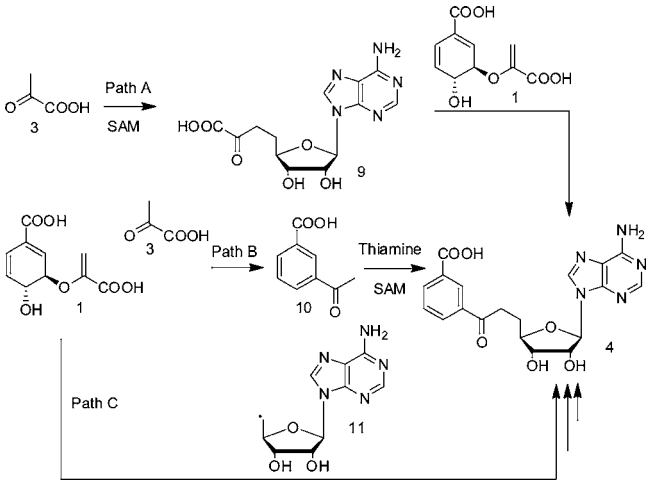

Figure 2. Three possible pathways for the formation of aminofutalosine (**4**) from chorismate (**1**).

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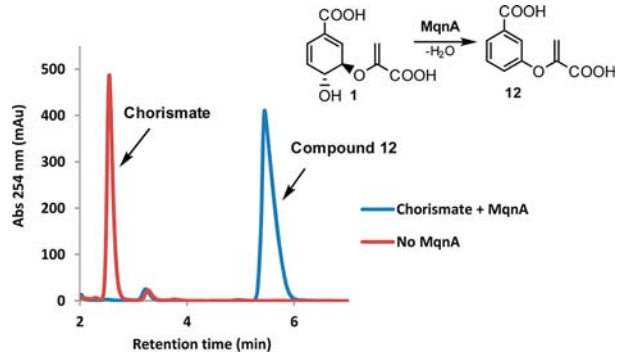

Figure 3. HPLC analysis of the reaction catalyzed by MqnA. Blue trace: chromatogram of the full reaction mixture (MqnA + chorismate). Red trace: chromatogram of the control reaction mixture lacking MqnA. The inset shows the structure of the identified MqnA reaction product, **12**, which was also characterized by 1D and 2D NMR (Figures S2–S6) and MS analysis (Figure S7).

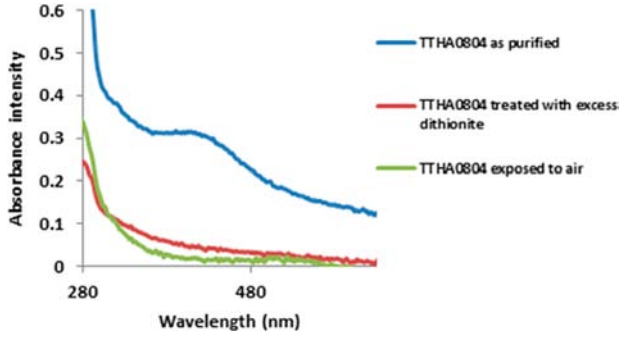

Figure 4. UV–visible spectra of MqnE (120 μM in each case). Blue trace: protein "as-isolated". Red trace: dithionite (excess) treatment of the "as-isolated" sample. Green trace: aerobic oxidation of the "as-isolated" sample.

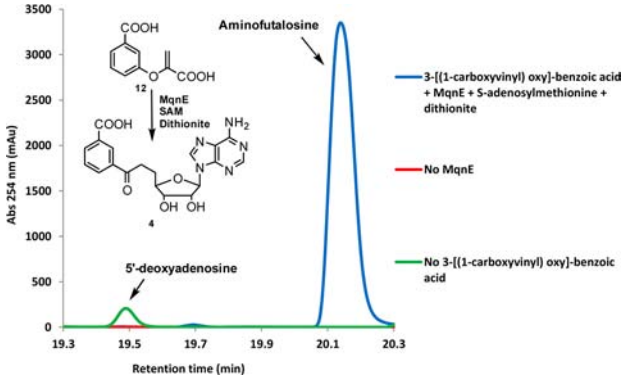

Figure 5. HPLC analysis of the MqnE reaction mixture. Blue trace: chromatogram of the full reaction mixture (MqnE + **12** + SAM + dithionite). Red trace: chromatogram of the control reaction mixture lacking MqnE. Green trace: chromatogram of the control reaction mixture lacking substrate **12**, showing low levels of the uncoupled production of 5′-deoxyadenosine. No signals at 20.2 and 19.5 min were obtained in the control reactions individually lacking SAM or dithionite (data not shown). The inset shows the structure of the identified MqnE reaction product, **4**, which was also characterized by 1D and 2D NMR (Figures S19–S23) and MS analysis (Figure S24).

putative ketoacid decarboxylases or a putative radical SAM enzyme using the SEED database (http://theseed.uchicago.edu/FIG/). This analysis yielded one promising gene (now

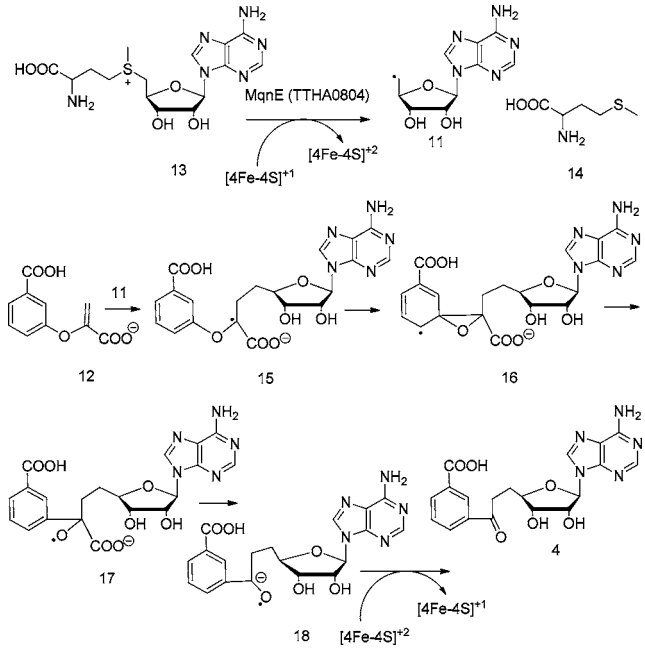

Figure 6. Mechanistic proposal for the MqnE-catalyzed formation of aminofutalosine (**4**).

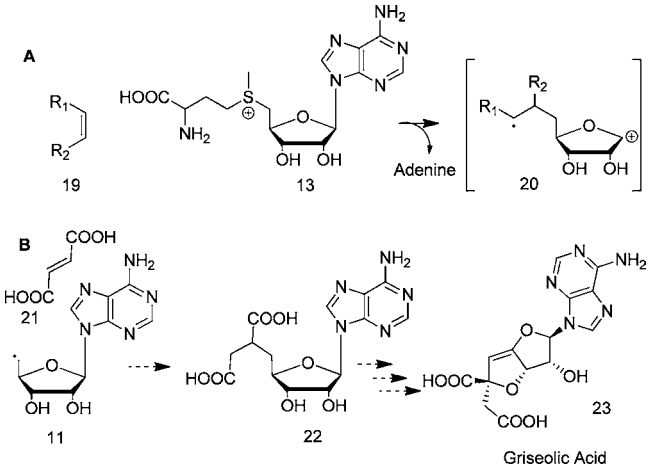

Figure 7. (A) Adenosyl radical addition to a double bond generates a new, potentially versatile biosynthon enabling chemistry at both C1 and C5 of ribose. (B) Griseolic acid biosynthesis presents a possible example of this chemistry.

named as *mqnE*), encoding a putative radical SAM enzyme that frequently clustered with menaquinone biosynthetic genes (SCO4494 in *S. coelicolor*, TTHA0804 in *Thermus thermophilus* HB8). As we were unable to find a similarly clustered putative ketoacid decarboxylase gene, we focused our attention on experimentally testing path C as the most likely route to **4**. In this Communication, we describe the successful reconstitution of aminofutalosine formation and demonstrate that it occurs by a new motif in radical SAM enzymology.

Genetic studies previously identified MqnA as essential for aminofutalosine formation.[6] To identify the reaction catalyzed by MqnA, the *mqnA* gene from *T. thermophilus* HB8 (TTHA0803) was amplified by PCR, cloned into the p28nT expression vector, overexpressed in *E. coli* BL21 (DE3), and purified by Ni-NTA affinity chromatography (Figure S1). Incubation of chorismate with MqnA resulted in the formation

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of a new product, which was identified as 3-[(1-carboxyvinyl)-oxy]benzoic acid (**12**) by NMR and MS analysis (Figures 3 and S2−S7). The steady-state kinetic parameters for MqnA are k_{cat} = 1.27 ± 0.02 s^{-1}, K_m = 161.6 ± 7.1 μM, and k_{cat}/K_m = 7.8 × 10^3 s^{-1} M^{-1} (Figure S17). To the best of our knowledge, MqnA is the first characterized enzyme catalyzing the dehydration of chorismate to give **12**.

To test the hypothesis that aminofutalosine (**4**) is formed via path C (Figure 2), *mqnE* (TTHA0804) from *T. thermophilus* HB8 was PCR amplified, cloned into the pQE-30 expression vector, and overexpressed in *E. coli* M15 (pREP4). The resulting His_6-tagged MqnE protein was purified by Ni-NTA affinity chromatography under anaerobic conditions (Figure S18). The purified protein had an absorbance maximum at 415 nm, which disappeared upon treatment with excess dithionite or on exposure to oxygen, consistent with the presence of the predicted [4Fe-4S] cluster (Figure 4). Iron and sulfide analysis yielded 2.4 irons and 2.8 sulfides per monomer of MqnE, indicating incomplete reconstitution of the cluster (Supporting Information).

To investigate the involvement of MqnE (TTHA0804) in the formation of aminofutalosine, 3-[(1-carboxyvinyl)oxy]-benzoic acid (**12**) was prepared by incubating chorismate with MqnA or by chemical synthesis (Figures S8−S16). Compound **12** (2 mM) was then incubated anaerobically with MqnE (200 μM) in the presence of *S*-adenosylmethionine (**13**, 2 mM) and excess sodium dithionite. After 10 h, the reaction was quenched with 8 M guanidine-HCl, protein was removed by ultrafiltration, and the reaction mixture was analyzed by reverse-phase HPLC. This analysis showed the formation of a new compound (Figure 5), which was identified as aminofutalosine **4** by 1D and 2D NMR (Figures S19−S23) and MS analysis (Figure S24). This identification was further confirmed by enzymatic conversion of the reaction product to futalosine using aminofutalosine deaminase[10] (Figures S25 and S26) and to compound **5** using MqnB[10] (Figures S27 and S28).

The MqnE reaction mixtures were also analyzed to determine if MqnE was catalytic and to determine the fate of the carboxylate of compound **12**. With the current enzyme preparations, 1 equiv of enzyme gives 14 equiv of the reaction product **4**, clearly demonstrating that MqnE is catalytic. NMR analysis of the reaction mixture, run using [^{13}C]-**12**, demonstrated the formation of bicarbonate and not formate, suggesting that the carboxy group of **12** is lost in a decarboxylation reaction (Figure S29).

A mechanistic proposal for the aminofutalosine synthase (MqnE)-catalyzed reaction is outlined in Figure 6. Addition of the adenosyl radical **11**, generated by the reductive cleavage of SAM **13**, to the vinyl double bond of compound **12** gives the captodative radical **15**. A similar radical addition has been reported in a pyruvate formate lyase-derived peptide in which the active-site glycine was replaced with dehydroalanine.[19] Rearrangement of this radical via **16** gives **17**. Such rearrangements have previously been proposed but not mechanistically characterized.[12−14] Decarboxylation of **17**, facilitated by the alkoxy radical, gives **18**. A final electron transfer from **18** to the $[4Fe\text{-}4S]^{2+}$ cluster completes the formation of aminofutalosine (**4**).

In summary, we have reconstituted the formation of aminofutalosine (**4**) in the menaquinone biosynthesis pathway. This reaction involves the addition of an adenosyl radical to the enol ether double bond of chorismate-derived **12** and represents a new catalytic motif in radical SAM enzymology, where all previously characterized enzymes[15−17] except for diphthamide synthase[18] use the adenosyl radical to abstract a hydrogen atom from the cosubstrate or from an active-site glycine. The MqnE-catalyzed reaction represents an efficient way to add adenosyl or ribose units to a double bond to generate an intermediate with rich biosynthetic potential, and other examples of this efficient catalytic motif are likely to emerge (Figure 7A). Griseolic acid biosynthesis presents one possible example in which intermediate **22** may be formed by addition of the adenosyl radical to fumaric acid[20,21] (Figure 7B).

■ ASSOCIATED CONTENT

Supporting Information

Detailed procedure for constructing the p28nT-*mqnA*; overexpression and purification protocols for MqnA, MqnE, SCO5662, and Hp0089; NMR and LC-MS characterization of 3-((1-carboxyvinyl)oxy)benzoic acid and aminofutalosine; and procedures for iron and sulfur determination in the purified TTHA0804. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

begley@chem.tamu.edu

Notes

The authors declare no competing financial interest.

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